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Brain Bioavailability Of Polyphenols: Implications For Delivery Of Brain Health Benefits

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BRAIN BIOAVAILABILITY OF POLYPHENOLS: IMPLICATIONS FOR DELIVERY OF BRAIN HEALTH BENEFITS

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This is for the Chen family.

You make this day possible for me.

Thank you.

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LIST OF ABBREVIATIONS

3’-OMeC-5-glucur: 3’-O-methylcatechin-5-glucuronide

3’-OMeEC-5-glucur: 3’-O-methylepicatechin-5-glucuronide

AD: Alzheimer’s disease

Am: amygdala

$AUC_{0\text{-}24h}$: area under the curve from 0 to 24h

$AUC_{0\text{-}8h}$: area under the curve from 0 to 8h

BW: body weight

C: catechin

C-5-glucur: catechin-5-glucuronide

CE: cerebellum

C_{max}: maximum plasma concentration

CNTL: control

Co: medial frontal cortex

Cy: cyanidin

Dp: delphinidin

EC: epicatechin

EC-5-glucur: epicatechin-5-glucuronide

GI: gastrointestinal

GSE: grape seed extract

HF: high fat

LC: liquid chromatography

LF: low fat

LN: lean

MS: mass spectrometry

MeO-Q-glucur: methylquercetin glucuronide

Mv: malvidin

Pn: peonidin

Pt: petunidin

Q: quercetin

Q-3-glucur: quercetin-3-glucuronide

Res: Resveratrol

Res-3-glucur: Resveratrol-3-glucuronide

SD rat: Sprague-Dawley rat

SGP: Standardized Grape Polyphenol

St: brain stem

T_{max}: time at maximum concentration

ZDF: Zucker diabetic fatty

ABSTRACT

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Consumption of fruits and vegetables has been associated with neuroprotection and cognitive benefits throughout the life span. These associations have sparked interest in plant-derived polyphenols as biologically active agents with potential for targeting brain benefits. However, little is known regarding the ability of the polyphenols or their metabolites from polyphenol-rich products to cross the blood-brain-barrier, and be available for biological action. Furthermore, additional insight is needed on factors affecting the absorption and brain distribution of polyphenol metabolites *in vivo*. To fill gap in current knowledge, this thesis will focus on the effects of diabetogenic diet and diabetic state which have been considered potential risk factors for neurodegenerative disease on polyphenol bioavailability from a 'Standardized Grape Polyphenol' (grape seed extract, Concord grape juice and resveratrol). Additionally, this thesis will provide novel evidence on plasma pharmacokinetics and regional brain distribution of polyphenol metabolites from apple/grape seed and bilberry extracts in a weaning piglet model.

We have found that background diabetogenic diet had limited effects on polyphenol plasma levels and brain bioavailability in a healthy Sprague-Dawley rat model. However, the diabetic state negatively influenced polyphenol metabolite levels in plasma and brain tissues, possibly in part, due to excessive excretion in urine. By using a young piglet model, we determined plasma pharmacokinetics of polyphenol metabolites using physiological and pharmacological doses of apple/grape seed and bilberry extracts. We found that in a physiological dose achievable by supplementation, polyphenol metabolites were able to cross blood-brain barrier to deposit in the brain. There was a difference in regional brain deposition with cerebellum being a preferred site for accumulation. Our data warrant the future design of functional tests on aging-related diseases, specifically Alzheimer's disease as well as improvement in cognitive and memory in healthy infant/child model.

CHAPTER 1. INTRODUCTION

1.1 Introduction

Consumption of polyphenol-rich foods are believed to offer health benefits for all age groups, including neuroprotective effects, especially against neurodegenerative diseases such as Alzheimer's disease (AD) in the elderly and, to a lesser extent, protection of early brain development in the young. Polyphenols rich food and supplements, in the form of grape seed extract (Wang, Ho et al. 2008), resveratrol (Vingtdeux, Giliberto et al. 2010) and red wine (Ho, Chen et al. 2009) have specifically demonstrated the ability to offer protection against the brain-aging processes leading to Alzheimer's disease in rodent models. On the other hand, evidence of polyphenol benefits on the young have been focused on various insults to the brain (Loren, Seeram et al. 2005; Candelario-Jalil, de Oliveira et al. 2007; West, Atzeva et al. 2007; Liu and Yu 2008; Narita, Hisamoto et al. 2011). Few have demonstrated hard data in support benefits to neuronal growth and brain development in healthy models (Patel, Scott et al. 2011). However, considering the potential for brain bioactive nature of these phytochemicals, it is not surprising that interest in research addressing the benefits for both young and old is growing. A key component to support such research is defining the bioactive polyphenol forms and

their ability to penetrate the blood-brain barrier and become available to brain tissues for action. It is crucial to understand polyphenol absorption and metabolism (bioavailability) in order to translate their physiological impacts of polyphenols into benefits in diseased as well as the healthy young population.

Several factors are known to impact polyphenol bioavailability including food processing (Ferruzzi 2010; Neilson and Ferruzzi 2011; Gupta, Kagliwal et al. 2013), food matrix (Scholz and Williamson 2007; Palafox-Carlos, Ayala-Zavala et al. 2011; Williamson 2013) and macronutrients composition (Ortega, Reguant et al. 2009; Jaldappagari, Balakrishnan et al. 2013). Specifically, fat content in the diet has increased dramatically over the years which has led to a severe obesity epidemic, furthering increasing development of metabolic syndrome and type II diabetes (NHANES 2010). The change in the dietary pattern toward a diabetogenic diet has been associated with the increased susceptibility to neurodegenerative disorders possibly due to the disruption of neuronal insulin metabolism (Pasinetti, Wang et al. 2011; Accardi, Caruso et al. 2012). Since we aim to target AD by the use of polyphenol-rich extracts, an understanding of the impact of dietary fat content on polyphenol absorption is needed for further therapeutic studies. Furthermore, habitual consumption of diabetogenic diet creates the conditions for the development of type II diabetes (Pasinetti, Wang et al. 2011). People with type II diabetes have double the risk for later dementia and Alzheimer's disease (Ott, Stolk et al. 1999; Willette, Xu et al. 2013). This relationship warrants the suitability of type II diabetes to be the model to investigate the effect of disease state on polyphenol bioavailability. Despite its much needed attention, the possibility that a diabetogenic

diet and the diabetic condition might influence polyphenol bioavailability has never been studied. In the elderly, polyphenols may be beneficial in prevention of neurodegeneration and improving and preserving cognitive function. In the young, the benefits may be linked to neuronal improvement. However, knowledge of polyphenols and brain health in healthy models is lacking. To gain more insight into the absorption and brain distribution of polyphenols from extracts in the healthy young population, we employed a piglet model. Overall, this dissertation will discuss the studies conducted aiming to obtain better understanding on the roles of polyphenol-rich products in AD prevention/treatment and young brain development. The data discussed merit the possibilities for further therapeutic studies regarding these potential polyphenol-rich extracts.

1.2 Outline and Organization

Chapter 2 will discuss the properties of key bioactive polyphenol compounds present in grapes, apples and berries, namely, flavan-3-ols, quercetin, anthocyanins and resveratrol and their benefits in young and old populations. Additionally, a summary of current findings of bioavailability studies and brain distribution of polyphenols as a function of different polyphenol dosages will also be highlighted. Chapter 3, 4 and 5 will highlight the investigation of the effects of diabetogenic diet and diabetic condition on polyphenol bioavailability in rodent models and the regional brain bioavailability in a young swine model with special focus on plasma pharmacokinetics and brain distribution.

CHAPTER 2. LITERATURE REVIEW

2.1 Classification of Polyphenols

Polyphenols are plant secondary metabolites produced, in part, as a self-defense mechanism to protect the plants from stress and disease including pathogenic attack, overexposure to ultraviolet radiation, and heat/drought (Davinelli, Sapere et al. 2012). ''Polyphenol'' is a general term for a broad class of compounds with chemical structures characterized by multiple aromatic/phenolic rings. Considering the broad possibilities for substitution to a basic phenolic structure, polyphenols have been broadly divided into two subgroups, flavonoids and non-flavonoids (Figure 2.1). Common dietary flavonoids are flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavan-3-ols. Non-flavonoids include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), lignans and stilbenes.

Flavonoids share the same C6-C3-C6 backbone with two aromatic rings A and B which are bound together by an oxygenated heterocyclic ring C illustrated in Figure 2.1 (Manach, Scalbert et al. 2004). The number and type of substitution of the backbone distinguishe one flavonoid from another. Polyphenol-rich foods like grape seed extract, Concord grape juice, resveratrol, bilberry and apple extracts used in the present research projects contains unique polyphenol subclasses. They are flavan-3-ols (catechin

and epicatechin), flavonol (quercetin), anthocyanidin glycosides and one non-flavonoid (resveratrol) which will be the main focus of this discussion with special focus on their distinct structure and major food sources.

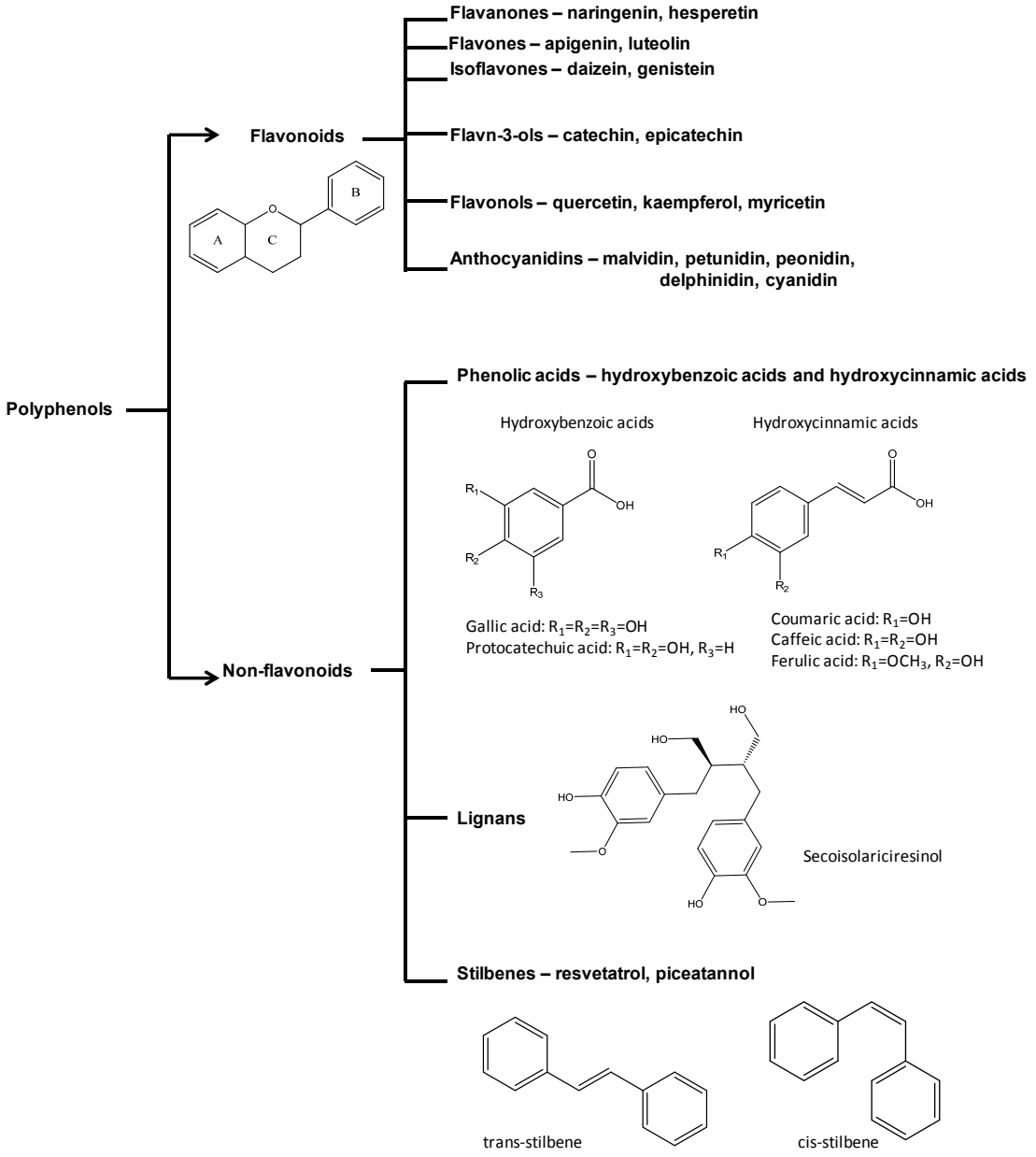


Figure 2.1. Classification of polyphenols.

2.1.1 Dietary Intake of Polyphenols

Estimation of polyphenol intake is important yet not readily accessible or likely accurate due to the difficulties in obtaining quantitative estimates of food content and consumption. The first report to ever document flavonoid intake, was released in 1976 and the estimation was 1g/d for U.S. adults (Kühnau 1976). Since then, average polyhphenol consumption of 1g/d has been considered to be the standard for polyphenol research. In 1995, the 'Seven countries study' was published documenting the average flavonoid intake of people from Greece, Italy, Finland, U.S., Japan, Netherland and former Yugoslavia (Croatia and Serbia) (Hertog, Kromhout et al. 1995). The report initiated studies to research polyphenol intake from different countries. However, the values reported in recent years were much lower than 1g/d estimated in 1976. The variations could be due to the methodology used such as dietary data acquisition, analytical methods, food and phenolic databases as well as the targeted polyphenol classes investigated. The background of each population chosen can contribute to the variation such as age, gender and health condition.

One of the most important factors is the inclusion of the numbers of flavonoids in the study. Most studies estimated flavonoid intake based on flavonols (kaempferol, myricetin and quercetin) and flavones (apigenin and luteolin) and some with the addition of flavanones (hesperetine and naringenin). The only report including all six flavonoids was by Chun et al. in 2007 estimating the average flavonoid intake of 189.7 mg/d for U.S. adults based on USDA database (Chun, Chung et al. 2007). Another factor

to consider is the age groups that were included in the studies. Most studies included subjects from 30 to 84 years old, two included all ages (Hertog, Hollman et al. 1993; Arts, Hollman et al. 2001) and two focused on elderly with age over 50 (Hertog, Feskens et al. 1994; Boker, Van der Schouw et al. 2002). Considering the possible different physiological impacts of flavonoids on the young and old, it is necessary to estimate the intake according to different age groups. Taken together, it is reasonable to assume that the average flavonoids intake of 189.7g/d reported by Chun et al. can represent flavonoid intake for U.S. adults (Chun, Chung et al. 2007).

While the physiological impacts on different age groups might be different, it is equally important to estimate flavonoid intake on the young and infants. There are limited data specific to young. None of these studies estimated total flavonoid intake of the young, but only focused on a specific flavonoid subgroup such as on proanthocyanidins (Gu, Kelm et al. 2004) or on anthocyanins (Drossard, Alexy et al. 2011). A summary of four current available data on polyphenol intake of infants and toddlers is shown in Table 2.1. The data on total flavonoid intake of the young including infants and children is much needed to connect the intake to physiological intakes for young age group.

Table 2.1. Polyphenol intake of infants and children.

Flavonoid Class	Age	Country	Flavonoid Database	Mean Intake (mg per day)	Primary form	Reference
Anthocyanins	3 mo 6 mo 9 mo 12 mo 18 mo 24 mo 36 mo	Germany	USDA 2007 (Release 2)	0.05 1.51 5.27 6.24 6.99 8.11 8.69	Total	Drossard et al. (2011)
	2-3 y 4-7 y 8-11 y	Australia	USDA 2003 (Release 1)	0.0 0.71 0.59	Cyanidin	Johannot & Somerset (2006)
Flavan-3-ols (monomer)	2-3 y 4-7 y 8-11 y	Australia	USDA 2003 (Release 1)	16.25 18.45 29.36	Catechins	Johannot & Somerset (2006)
	4-6 m 6-10 m 2-5 y 6-11 y	USA	Calculated	0.2 2.3 3.9 4.4	Monomers	Gu et al. (2004)
Flavonols	2-3 y 4-7 y 8-11 y	Australia	USDA 2003 (Release 1)	3.11 3.63 4.19	Quercetin Kaempferol	Johannot & Somerset (2006)

Adopted from Ferruzzi 2010.

2.1.2 Flavan-3-ols

Structure - Flavan-3-ols, also referred to as catechins, is one of the main subclasses of flavonoids. They have the backbone structure of diphenylpropane (C6-C3-C6) which contain a 3-carbon ring in the middle and two benzene rings with two hydroxyl groups on both sides of the C3 ring (Song and Chun 2008) (Figure 2.2). The major flavan-3-ols includes (-)-epicatechin (EC), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC) and (-)-epigallocatechin-3-gallate (EGCG) with structures shown in Figure 2.2. The backbone structure contains two chiral carbons which generates two diastereoisomers namely catechin and epicatechin derivatives which are monomeric units of the flavan-3-ol structure. The single unit (monomer) makes the building block for polymers called

complex proanthocyanidins. Monomers, catechins and epicatechins, abundant in grape seed extract will be the focus in this discussion.

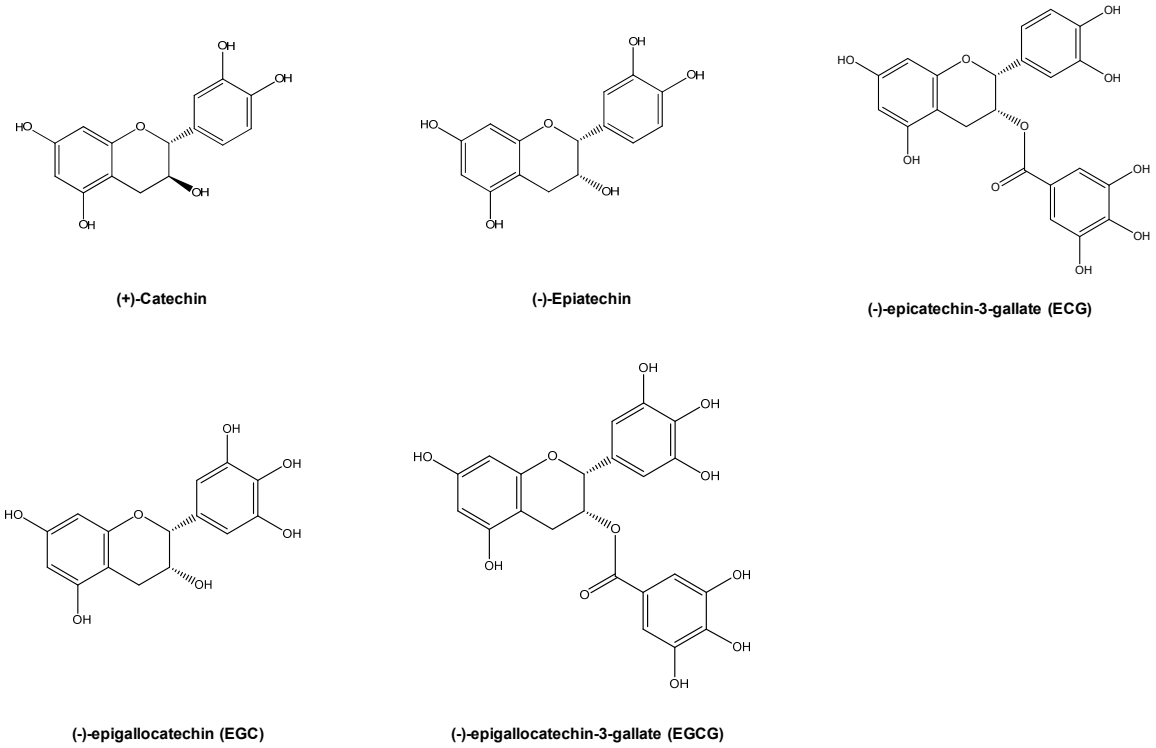


Figure 2.2. Structures of flavan-3-ols.

Food source - Flavan-3-ols present in plants and foods in their free forms (EC) or as gallic acid esters to form ECG, EGC and EGCG. According to the USDA data on 225 foods (USDA 2003), dark chocolate contains the most catechin and epicatechin. Apple with skin contains 0.95mg of catechin per 100g dry weight and 8.14mg of epicatechin per 100g dry weight. Another reference indicates that the number one source of flavan-3-ols is tea, second is wine, third is apple (Song and Chun 2008). There are many studies which determined catechin amounts in different sources of foods from different origins. For example, Pascual-Teresa et al. evaluated different Spanish foodstuffs including three

apple cultivars in which Red Delicious apples contained the highest amount of 1.57mg of catechin/100g fresh weight and 6.37mg epicatechin/100g fresh weight (de Pascual-Teresa, Santos-Buelga et al. 2000). Red grape contained 0.82mg of catechin/100g fresh weight and 0.7mg of epicatechin/100g fresh weight (de Pascual-Teresa, Santos-Buelga et al. 2000). Arts et al. measured flavan-3-ol contents in different red wines, white wines and other beverages. They showed that Bordeaux (Appellation Controlee, Rineau, 1997) contained the highest amount of catechin (53.4mg/L) and epicatechin (42.1mg/L) (Arts, 2000) in the red wine category. Bordeaux Sauvignon (Appellation Controlee, Euroshopper, table wine) ranked number one in the white wine category with 4.8mg of catechin/L and 1.2mg of epicatechin/L (Arts, van De Putte et al. 2000). A summary of catechin and epicatechin contents derived from USDA data is shown in Table 2.2.

Table 2.2. Summary of catechin and epicatechin contents in selected foods.

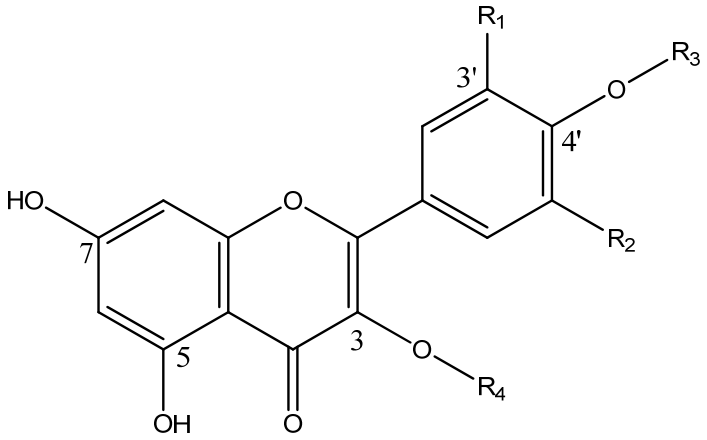
	Content (mg/100g)	
Food Source	(+)-Catechin	(-)-Epicatechin
chocolate bar, dark	11.99	41.5
chocolate bar, milk	2.9	10.45
Apple, with skin	0.95	8.14
Apple, no skin	0.86	6.23
Grape juice, canned or bottled	0.19	0
Grape, red	NA	1.95
Grape, black	8.94	8.64
Bilberry, raw	NA	NA
Onion, cooked	NA	NA
Onoin, raw	NA	NA
Tea, black, brewed	1.52	2.33
Tea, green, brewed	2.73	8.47
Wine, red	7.61	4.29

USDA data was generated by surveying the scientific literature on 225 foods with flavonoid contents. Data generated only by HPLC can be accepted in the USDA flavonoid database (USDA 2003).

2.1.3 Flavonols (Quercetin)

Structure - The most common flavonols are myricetin, quercetin and kaempferol, structures are shown in Figure 2.3. The main flavonol investigated in this dissertation was quercetin due to its abundance in Concord grape juice, wine and the apple extract utilized in the current research (to be discussed later). Quercetin, like flavan-3-ols, consists of two phenolic rings linked by a heterocyclic pyran ring in the middle (Harwood,

Danielewska-Nikiel et al. 2007). Generally, flavonols exist in plants in conjugated forms with sugar moiety attached to the 3-position of the unsaturated C-ring forming O-β-glycosides like quercetrin or rutin shown in Figure 2.3 (Harwood, Danielewska-Nikiel et al. 2007). The glycosylated flavonols are therefore the most common structure seen in plants, not the aglycone or free flavonoid backbone structure (Murota and Terao 2003).



Flavonols	**R_1**	**R_2**	**R_3**	**R_4**
Quercetin	-OH	-H	-H	-H
Kaempferol	-H	-H	-H	-H
Myricetin	-OH	-OH	-H	-H
Derivatives				
Rutin	-OH	-H	-OH	Rutinose
Quercetrin	-OH	-H	OH	Rhamnose

Figure 2.3. Structures of major flavonols and sugar derivatives.

Food source - Quercetin is widely distributed in the human diet in various foods and has been shown to be one of the most prevalent flavonoids consumed (Hertog, Hollman et al. 1993; Hertog, Feskens et al. 1994; Knekt, Jarvinen et al. 1996; Rimm, Katan et al.

1996; Arai, Watanabe et al. 2000). Like other flavonoids, quercetin content in plants varies greatly due to climate, cultivar, disease pressure, post-harvest handling and processing, food preparation and also storage conditions. Key dietary sources of quercetin include onion (347mg/kg), apple (36mg/kg), black tea (20mg/kg) (Hertog, Hollman et al. 1993). Quercetin in Spanish cherry tomatoes was found to be 55mg/kg, in Lollo Rosso lettuce 911mg/kg and in yellow onions 1359mg/kg (Crozier, Burns et al. 2000). A more extensive list of quercetin content in foods determined by HPLC was composed by USDA (USDA 2003) and revised for a representative summary in Table 2.3. The summary table depicts most relevant food sources used in our research studies including apple, grape and bilberry products and grape juice. Onions, tea and wine are the most commonly consumed quercetin-rich products to provide a reference for comparison.

Accumulation of quercetin derivatives by specific portions of plant tissues has been studied. For example, a study compared flavonoid content in the flesh and in the peel of an apple and confirmed that peel had higher levels of quercetin than the flesh (USDA 2003; Lamperi, Chiuminatto et al. 2008). Crozier et al. compared tomatoes, lettuce, onions and peas from different varieties and found an obvious variation in quercetin concentration in different cultivars (Crozier, Burns et al. 2000). Nemeth et al. summarized in a review that different research groups yielded different results on quercetin derivative contents on the same variety of onion (Nemeth and Piskula 2007). Overall, based on the USDA data from 225 foods, food sources with most abundant

quercetin content except for dry tea leaves are canned capers (180mg/100g), fresh dill weed (55mg/100g) and yellow wax hot peppers (50mg/100g) (USDA 2003).

Table 2.3. Summary of quercetin content in selected foods.

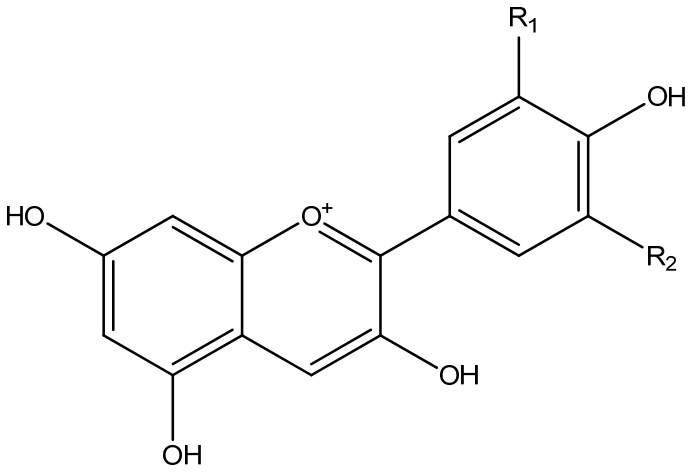
Food Source	Quercetin aglycon content (mg/100g)
Capers, canned	180.77
Apple, with skin	4.42
Apple, no skin	1.5
Grape juice, canned or bottled	0.41
Grape, red	3.54
Grape, black	2.54
Bilberry, raw	3.04
Onion, cooked	19.36
Onoin, raw	13.27
Tea, black, brewed	2.07
Tea, green, brewed	2.69
Wine, red	0.84

USDA data was generated by surveying the scientific literature on 225 foods with flavonoid contents. Data generated only by HPLC can be accepted in the USDA flavonoid database (USDA 2003).

2.1.4 Anthocyanidins

Structure – The five common anthocyanidin structures are shown in Figure 2.4. In aquesous solution, a positive charge on the heterocyclic oxygen ring is responsible for the red to blue color shown under acidic pH. Structures of anthocyanidins change depending on environmental pH, enzymatic reactions, light, temperature and exposure to oxygen (Yang, Koo et al. 2011). Anthocyanidins rarely exist in nature due to poor

stability and are stabilized by glycosylation into glucosides (anthocyanins) or other glycosylated forms (Wallace 2011). There has been over 635 anthocyanins identified with six major glycosylated anthocyanidins including cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin which account for over 90% occurring in nature (Wallace 2011). Anthocyanins occur more frequently as 3-glucosides, 3-diglucosides and sometimes 3-triglucosides, 3,5-diglycosides and 3,7-diglycosides. They are also found to be glycosylated with sugars like galactose, rhamnose, arabinose and xylose (McGhie and Walton 2007).



Anthocyanidins	R_1	R_2
Cyanidin (Cy)	-OH	-H
Malvidin (Mv)	$-OCH_3$	$-OCH_3$
Delphinidin (Dp)	-OH	-OH
Peonidin (Pn)	$-OCH_3$	-H
Petunidin (Pt)	$-OCH_3$	-OH

Figure 2.4. Structures of major anthocyanidins.

Food source - Anthocyanins are largely present in berries which are extensively incorporated in the diet. The mean intake of anthocyanins in Finland has been estimated to be 82mg/d with the main sources being berries, red wine and juice (Manach, Williamson et al. 2005). Table 2.4. provides a representative summary of the major anthocyanin contents in common foodstuffs analyzed by a standardized HPLC method (USDA 2003).

Table 2.4. Summary of major anthocyanin content in selected foods.

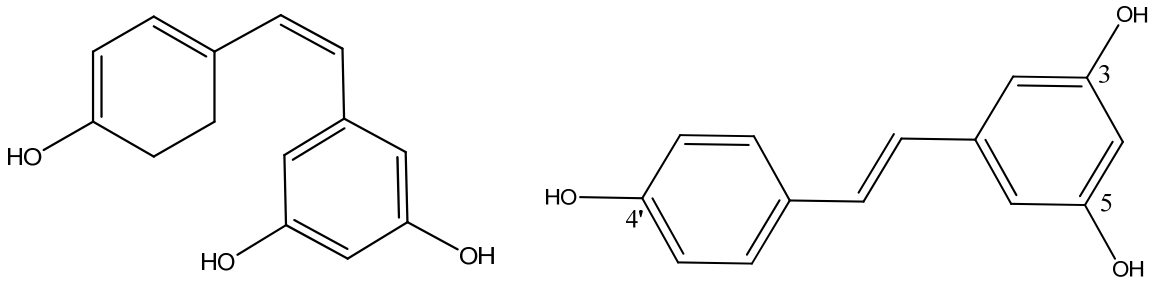
	Content (mg/100g)				
Food Source	Cy	Dp	Mv	Pn	Pt
Grape juice, canned or bottled	NA				
Grape, red	NA				
Grape, black	NA				
Bilberry, raw	15.02	29.54	49.21	7.05	11.73
Wine, red	0.27	0.68	5.68	1.17	1.39

[1]USDA data was generated by surveying the scientific literature on 225 foods with flavonoid contents. Data generated only by HPLC can be accepted in the USDA flavonoid database (USDA 2003).
[2]Abbreviations: cyanidin (Cy), delphinidin (Dp), malvidin (Mv), peonidin (Pn) and petunidin (Pt). Not available (NA).

2.1.5 Resveratrol

Structure - Resveratrol (3, 5, 4' – trihydroxystilbene) is a phenolic compound in the stilbene family. Resveratrol exists in two structural isomeric forms, *cis* and *trans* (Figure 2.5.) with *trans* form being more common in food stuffs and possessing greater bioactivity (Wenzel and Somoza 2005). Resveratrol was first discovered in high abundance by Japanese scientists in the early 1980 in the roots of Polygonum

cuspidatum (Arichi, Kimura et al. 1982). Resveratrol was later hypothesized to be the component of red wine responsible for the French Paradox, which is the lower risk of coronary heart disease in France, despite their diet high in saturated fat (Renaud and de Lorgeril 1992).



Cis-resveratrol **trans-resveratrol**

Figure 2.5. Structures of *cis*-resveratrol and *trans*-resveratrol.
Positions denoted with numbers are sites of glucuronidation and/or sulfation in biological matrices.

Food source - Resveratrol is found in over 72 plants species but the primary dietary sources are grape derived products including selected juices and red wines, dark chocolate, select berries, pistachio, peanuts and peanut products (King, Bomser et al. 2006; Fernandez-Marin, Mateos et al. 2012). The concentration of resveratrol present in products is dependent on several factors such as cultivar, climate, geographic region and also different components of a fruit. For example, the content of *trans*-resveratrol in different red wines were reported to range from undetectable to 14.3mg/L (Stervbo, Vang et al. 2007; Fernandez-Marin, Mateos et al. 2012) while others reported to range from 0.1 to 15mg/L (Fremont 2000). As an example of the variability of resveratrol

content in different components of fruit, resveratrol is found in the seed and skin of grapes but not in flesh and therefore, relatively high concentrations were found in red wine fermented with flesh and skin while white wine which uses only the flesh of the grape had little or no resveratrol (King, Bomser et al. 2006).

The dietary intake of flavonoids are low as 100mg/d as discussed previously. The major food sources contain low levels of flavonoids: <50mg/100g food weight (USDA 2003). Since the dietary levels of flavonoids are so low, it is reasonable to increase the intake with dietary supplements where these can be shown to have health benefits. According to World Health Organization (WHO), ~80% of the world population use botanicals for health. About 42% of population in the United States claimed to have used botanical dietary supplements to improve health status (Khan and Smillie 2012). The increasing prevalence polyphenol supplement use has resulted a huge revenue increase for the dietary supplement industry to $4.8 billion in 2008 compared to only $2.9 billion in 1995 (Khan and Smillie 2012). However, one botanical plant may contain different compounds and many of them could contribute to the health benefits. In order to gain more insights on the specific bioacatives responsible for their neuroprotective actions, more studies need to be conducted to identify the active compounds in foods as well as the dietary effects. The studies discussed in this dissertation were designed based on the idea described here aiming to identify the bioavailability of polyphenols from grape-derived extracts and from some other commonly consumed fruit extracts. The popularity of botanicals on their therapeutic effects warrant the investigation on

the absorption and metabolism of polyphenol-rich botanicals to further support the usage of dietary supplements of naturally occurred botanicals for general health.

2.2 Food Matrix and Polyphenol Bioavailability

Many factors have been suggested to influence the absorption of flavonoids including insufficient absorption in gastrointestinal tract, interaction with the food matrix and the influences of food processing (Ferruzzi 2010). We focus in this section on the interaction of flavonoids and food matrix since we are interested in the impact of high-fat diabetogenic diet on polyphenol absorption. There have been many studies conducted to understand the interaction of protein and polyphenols absorption (see review in (Bandyopadhyay, Ghosh et al. 2012) and (Ferruzzi 2010)). However, there has been very few investigation of the effects of fat content on polyphenol absorption. When searching Pubmed with key words combinations of 'fat content', 'polyphenol absorption', 'bioaccessibility and bioavailability', there was only one study that investigated the effect of fat content on cocoa polyphenol bioaccessibility in an *in vitro* digestion model (Ortega, Reguant et al. 2009). They showed that high fat content in cocoa liquor protected catechins during digestion and therefore demonstrated better uptake in the enterocytes. The lack of data on how fat content can influence polyphenol absorption is possibly due to the hydrophilic nature of flavonoids (Scholz and Williamson 2007) and therefore, their interaction with fat to form micelles is limited. It is necessary

to obtain data on the impact of fat content on polyphenol absorption since fat is the major fraction in modern western diet.

2.3 Polyphenols and Neurodegenerative Diseases

Increasing evidence suggests that plant-derived polyphenols play an important role in improving cognitive functions and preventing/delaying the onset of certain neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease. Our project specifically focused on Alzheimer's disease since it is the most common neurological disorder affecting 18 million worldwide and estimated to reach 34 million by 2025 (Mount and Downton 2006). The association between polyphenol consumption and lower AD risk is based on several epidemiological studies. A prospective study published in 1997 in the Bordeaux area in France concluded that moderate wine consumption (3 to 4 glasses per day) negatively correlated to AD risk (Orgogozo, Dartigues et al. 1997). The Copenhagen City Heart study concluded that monthly and weekly wine consumption was associated with a lower risk of dementia, however, the volume of the drinks was not specified (Truelsen, Thudium et al. 2002). Many prospective studies subsequently confirmed a negative relationship between flavonoids intake and incidence of dementia or AD. For example, the Letenneur group in the Personnes A'gees Quid study concluded that high flavonoid intake (the highest quartile: 17.7 – 36.94 mg/d) correlated with better cognitive ability over a 10-year tracking period (Letenneur, Proust-Lima et al. 2007). Scarmeas and colleagues published

a series of studies validated the relationship between a Mediterranean diet and risk for AD (Scarmeas, Stern et al. 2006; Scarmeas, Stern et al. 2009). In 2006, they concluded that higher adherence to the Mediterranean diet is associated with a lower risk for AD in a community-based study conducted in New York City with a 4 year follow-up (Scarmeas, Stern et al. 2006). In 2009, they further concluded that higher adherence to Mediterranean diet also reduced risk of developing mild cognitive impairment which later progress to AD in a similar setting to the previous report in 2006 (Scarmeas, Stern et al. 2009). Although there is still significant debate on the potential benefits of polyphenol with regard to age-related diseases, evidence appears to be growing for these plant derived compounds as promising dietary/supplemental neuroprotective agents.

2.3.1 Type II Diabetes and Alzheimer's Disease

Due to the multifaceted nature of AD, the primary cause and pathways to a cure for AD remain elusive. Researchers have focused on possible factors such as genetics, nutrition and lifestyle that may influence the risk for AD to prevent as well as to develop strategies for treatment of AD. Among all the metabolic disorders, type II diabetes disturbs systemic and central metabolic functions which directly influences glucose metabolism in brain (Kahn, Hull et al. 2006). The relationship between type II diabetes and AD has been documented, but remains controversial. For example, researchers found a two fold increase in risk for AD in men with diabetes and ~1.5 fold in diabetic

women compared to the non-diabetics in a cohort in Rochester, MN (Leibson, Rocca et al. 1997). The Rotterdam study yielded similar results that diabetic patients had two times higher risk for dementia and AD (Ott, Stolk et al. 1999). A review by Cukierman et al. summarized 25 prospective studies with more than 8656 people with diabetes in a follow-up period ranged from 2 to 18 years and the authors found a 1.5-fold increase in risk for cognitive decline and a 1.6-fold greater risk of future dementia in diabetics compared to the non-diabetics (Cukierman, Gerstein et al. 2005). Recently, a studied employed MRI to determine grey matter volume in a middle-aged cohort and found that the level of insulin resistance positively correlated with brain atrophy (Willette, Xu et al. 2013). The strong correlation between type II diabetes and AD suggest that type II diabetes may in fact play a role in onset or progression of this disease. Therefore, the impact of type II diabetes on polyphenol bioavailability and metabolism should be better understood.

2.3.2 Mechanism of Action of Polyphenols in Alzheimer's Disease

There is considerable evidence for the benefits of polyphenol-rich foods and dietary supplements in the prevention of age-related neurodegenerative diseases (see review in (Ebrahimi and Schluesener 2012). While specific mechanisms remain elusive, it has been proposed that protection of age-related diseases by polyphenols may be due, in part, to their properties of antioxidants, increase of nitric oxide production and anti-inflammation mechanisms (Campos-Esparza Mdel and Torres-Ramos 2010; Albarracin,

Stab et al. 2012). It has been proposed that neuroinflammation and neurotoxicity are the two major causes of neuronal dysfunction. Each of them includes several toxic phenomena which play important roles in Alzheimer's disease pathogenesis. These toxic phenomena are also the important targets for polyphenols in the prevention of most neurodegenerative disease which will be described in the following paragraphs.

Neuroinflammation - Inflammation is a defense response of cells to insults like toxins, tissue injuries or Aβ in neuroinflammation. The transient neuroinflammation response starts with microglia activation. Activated microglia function as macrophages in the central nervous system (CNS) but engulfing Aβ by phagocytosis (Querfurth and LaFerla 2010) or binding Aβ by the receptor for advanced glycation endproducts (RAGE) (Gu, Kelm et al. 2004). Prolonged Aβ insults result in long-term microglia activation which induces the release inflammatory mediators including chemokines and cytokines such as IL-1β, IL-6, TNF-α and chemokine IL-8. Release of inflammatory cytokines induces more microglia activation which eventually results in neuronal cell death (Lee, Rizer et al. 2010). The astrocyte is another key player in the neuroinflammatory process. Astrocytes have been show to express RAGE which bind Aβ and further degrade Aβ (Wyss-Coray, Loike et al. 2003). A study showed that activated astrocytes induced by Aβ also release chemokines and cytokines which promote neuron death (Smits, Rijsmus et al. 2002). Overall, when facing Aβ attacks, microglia and astrocytes work together on Aβ clearance and degradation. However, ineffective Aβ clearance leading to the release of

inflammatory mediators is thought to be the dominant process, leading to the accelerated development of AD (Fiala, Lin et al. 2005).

Neuroinflammation results in an increase in nitric oxide, and cytokine production. These processes are enhanced by the presence of APOε4 genotype. Polyphenols modulate neuronal dysfunction by targeting these mechanisms which contribute to neuroinflammation. It has been demonstrated that quercetin inhibits neuroinflammation by decreasing nitric oxide and cytokine production in microglia (Sharma, Mishra et al. 2007; Bureau, Longpre et al. 2008; Kao, Ou et al. 2010). Catechin and epicatechin were shown to prevent TNF-α release in primary glial cells at a physiological relevant doses (10-300 nM) (Vafeiadou, Vauzour et al. 2009). Blueberry polyphenols were shown to inhibit nitric oxide, IL-1β and TNF-α production induced by lipopolysacharides in microglia cells (Lau, Bielinski et al. 2007).

Neurotoxocity – Neurotoxinic mechanisms such as oxidative stress, protein phosphorylation, protein aggregation are thought to contribute to neuropathogenesis. The health benefits of polyphenols are thought to be due to their free radical scavenging capacity imparted by the phenol structure of the flavonoid backbone (Visioli, Bellomo et al. 1998; Russo, Acquaviva et al. 2000; Halliwell 2006). Recently this hypothesis has been challenged due to extremely high concentrations of polyphenols are required to show desired benefits {Williams, 2004 #85}. A high plasma or tissue concentration of polyphenols often times is difficult to achieve *in vivo* due to the low bioavailability and high excretion rate of most dietary polyphenols. Therefore, although polyphenols have

antioxidants capacity, their potential impact may be reduced due to low bioavailability (Williams, Spencer et al. 2004). In the case of Alzheimer's disease, tau protein phosphorylation is thought to contribute to neurodegeneration. The normal function of tau is to stabilize microtubules of neurons. However, hyperphosphorylated tau protein has low infinity to microtubules and therefore easily self assembled (Ballatore, Lee et al. 2007). Aggregation of hyperphosphorylated tau form neurofibrillary tangles (NFTs) which are toxic and can lead to neuron death (Roy, Cohen et al. 2005). It has been suggested that polyphenols may inhibit tau protein aggregation. For example, grape polyphenols has been shown to attenuate tau neuropathology in a transgenic Alzheimer's mouse model (Wang, Santa-Maria et al. 2010). EGCG has been shown to modulate tau pathology in a transgenic Alzheimer's mouse model (Arendash, Mori et al. 2009).

Protein aggregation is another toxic phenomenon that play an important role in AD pathogenesis. Aβ is thought to be the cause to neuronal loss which leads to dementia and other neurodegenerative disorders. Aβ is a peptide with 40 or 42 amino acids. Aβ42 with hydrophobic amino acids at the sequence tail induce cell death due to this unique chemical structure .Aβ peptides first form soluble prefibrillar oligomers and further aggregate to prefibrillar oligomers and to fibrils and finally to insoluble plaques (Sakono, Zako et al. 2008). Prefibrillar oligomers are considered to be more toxic than plaques (Caughey and Lansbury 2003; Ferreira, Vieira et al. 2007). There is mounting evidence that polyphenols inhibit Aβ deposition decreasing AD pathology in *in vivo* or *in vitro*

models. For example, grape seed extracts consumption has been shown to prevent Aβ deposition in the brain of Alzheimer's disease mice (Wang, Thomas et al. 2009). Polyphenols in Cabernet Sauvignon have been shown to inhibit Aβ aggregation in am AD mouse model (Ho, Chen et al. 2009).

2.3.3 Type II Diabetes and Polyphenol Metabolism

Type II diabetes is known to alter key endogenous hormone secretion including insulin and glucagon (Samsom, Vermeijden et al. 2003). Insulin resistance and hyperglycemia lead to changes in expression of certain phase II metabolizing enzymes (Kim and Novak 2007). For example, researchers showed increased gene expression of UDP-glucuronosyltransferase (UGT 1A1) in a diabetes rat model (Tunon, Gonzalez et al. 1991; Braun, Coffey et al. 1998) but decreased of sulfotransferase (SULT) 2A1 (Runge-Morris and Vento 1995). Polyphenols are substrates for UGT and SULT during glucuronidation and sulfation process and therefore, disturbance in enzyme gene expression might lead to changes in generation of biologically relevant polyphenol metabolite levels. Some of the most common complications of type II diabetes are gastrointestinal (GI) symptoms such as vomiting, constipation, and diarrhea (Feldman and Schiller 1983). The GI irregularities were thought to be due to abnormal GI motility in diabetic patients. Feldman et al. demonstrated that diabetic patients had delayed gastric empty compared to non-diabetics (Feldman and Schiller 1983). The delayed gastric emptying of liquid and solid foods was later validated by other researchers

(Horowitz, Edelbroek et al. 1991; Samsom, Vermeijden et al. 2003). Delayed gastric emptying may influence oral drug absorption (Horowitz, O'Donovan et al. 2002) and therefore, possibly affect polyphenol absorption and bioavailability. Considering the strong link between metabolic dysfunction in type II diabetes and neurodegenerative diseases, it is important to understand the impact of diabetic condition on polyphenol bioavailability and metabolism in order to develop optimal dosing strategies for prevention or therapy.

2.4 Polyphenols and Brain Development in Infants and Children

Evidence for the benefit of polyphenols-rich foods and dietary supplements in prevention of age-related neurodegenerative diseases is substantial (Ebrahimi and Schluesener 2012). Two plausible underlying mechanisms have been previously discussed. With underlying mechanisms of protection also related to improvement/preservation of cognitive function, interest in the application of similar polyphenols to promote healthy brain and cognitive development in young population has grown. However, few studies exist on polyphenol benefits in young populations. Most investigations have focused on prenatal diseases when addressing the beneficial effects of polyphenols in early life. For example, Loren et al. showed that maternal supplementation with pomegranate juice protected against hypoxic-ischemic injury in rat pup's brains (Loren, Seeram et al. 2005). Narita et al. demonstrated that grape seed extract protected glutamate-induced insults in a primary neonatal mouse neuron

culture (Narita, Hisamoto et al. 2011). Few have discussed the effects of polyphenols on healthy normal neurons. After searching in PubMed with the combination of key words like ‘polyphenol’, ‘neonate’, ‘young’, ‘infant’, ‘neuron’, ‘cell growth’, there are only two papers out of 49 relevant to normal cell growth and polyphenols (Godlewski, Slazak et al. 2006; Han, Lee et al. 2011). Han et al. found that EGCG offered protection to normal human dermal fibroblasts (Han, Lee et al. 2011). Godlewski et al. reported that boiled soybeans increased mitotic ratio in the intestinal mucosa of young rats which suggested faster cell turnover of enterocytes (Godlewski, Slazak et al. 2006). No direct publication is found under Pubmed search on polyphenols and healthy neuron growth. More studies are needed to better understand the influences of polyphenols on young developing brain.

2.5 Polyphenol Absorption, Metabolism and Excretion

After ingestion, polyphenols enter gastrointestinal tract to undergo absorption, metabolism and excretion. Flavan-3-ols, quercetin and anthocyanins are absorbed through shared mechanisms but with minor differences due to their chemical structures which directly influence their potential to circulate in plasma and to reach target tissues. Their differences in bioavailability may be linked to their ability to influence brain health and development. Additionally, flavonoids are metabolized extensively leading to the structural modifications (conjugations) which influence tissue distribution and excretion. Finally, polyphenols and/or their metabolites are excreted from the body and some can

be further metabolized in colon by colonic microflora. The following section will discuss the absorption, metabolism and excretion of flavan-3-ols, quercetin and anthocyanins. The proposed polyphenol absorption and metabolism mechanism is illustrated in Figure 2.6.

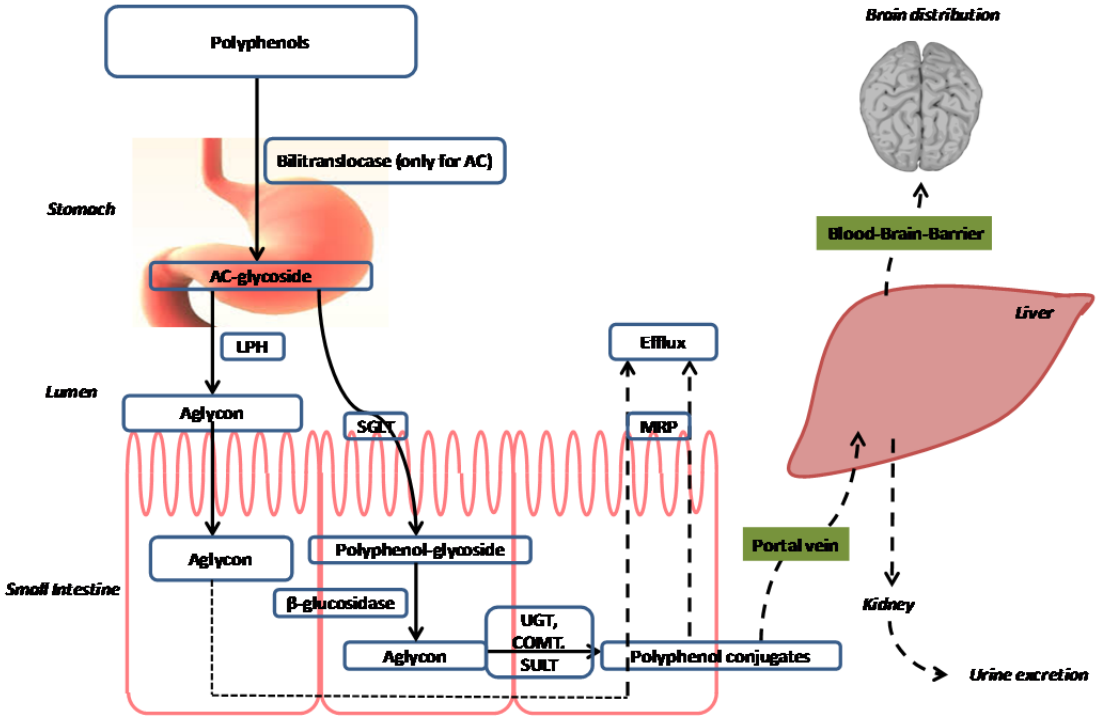


Figure 2.6. Proposed polyphenol absorption and metabolism pathway.

2.5.1 Absorption

Stomach- Following oral processing, flavonoids in foods and beverages are digested in the gastric environment. There is little to no evidence that flavan-3-ols and quercetin are absorbed in the gastric phase. However, they are believed to be stable in this environment and begin to be extracted from the food matrix as the digestion of food

facilitates release of flavonoids (Green, Murphy et al. 2007). Anthocyanins however, have been shown to be readily absorbed in the gastric environment through the stomach. It was reported that after 2.7mg of cyanidin-3-glucoside consumption, human plasma reached 24 nmol within 30 minutes indicating the absorption taken place in the stomach (Miyazawa, Nakagawa et al. 1999). It was also shown in an anesthetized rat model that anthocyanin glucosides are absorbed in the stomach (Passamonti, Vrhovsek et al. 2003). The mechanism on gastric absorption of anthocyanins has been suggested to involve active transport through the bilitranslocase transporter in the stomach (Passamonti, Vrhovsek et al. 2002). After absorption intact from the stomach, anthocyanins (primarily glucosides) move into the blood and are distributed to tissues (described later). Unabsorbed fraction of anthocyanins and other flavonoids are emptied into the small intestine where digestion and continues.

Small intestine - Unlike other flavonoids usually in glycosylated forms, flavan-3-ols generally exist as aglycones. Once ingested, flavan-3-ol monomers can be directly absorbed into enterocytes by active uptake for further transformation (Monagas, Urpi-Sarda et al. 2010). There are two major pathways being suggested for quercetin glucoside absorption. One pathway is that quercetin-4'-glucoside, quercetin-3-glucoside and quercetin-3,4'-glucoside were deglycosylated by lactase phloridzin hydrolase (LPH) in the small intestinal lumen to become quercetin aglycone which further diffuse into enterocytes by passive transport due to its lipophilic structure in a *in vitro* model (Day, Canada et al. 2000). The LPH pathway was also observed in the Caco-2 cell model

(Murota, Shimizu et al. 2002). The other pathway being suggested was that quercetin glycosides are transported into the enterocytes by sodium-dependent glucose transporter (SGLT-1) (Hollman, de Vries et al. 1995) and deglycosylated by the cystolic β-glucosidase to form quercetin aglycones (Day, DuPont et al. 1998; Crespy, Morand et al. 2001) in rats and humans. It has been shown in a Caco-2 cell model that quercetin glycosides were the substrates for multi-drug-resistance protein 2 (MRP2) which are responsible for effluxing quercetin glycosides out to the intestinal lumen (Walgren, Karnaky et al. 2000). Based on that observation, the LPH pathway is considered to be the primary mechanism for quercetin to be absorbed into the enterocytes. After gastric absorption in the stomach, anthocyanins continue to be absorbed in the small intestine in a rat model (Talavera, Felgines et al. 2004) and a Caco-2 cell model (Yi, Akoh et al. 2006). There is no consensus in terms of the mechanism as to how anthocyanins are absorbed. LPH and SGLT-1 pathways similar to quercetin were proposed by Kay et al. (Kay 2006) which contradicted by the evidence provided by Walton et al. in a mice model (Walton, McGhie et al. 2006). More studies are needed to gather more data. Resveratrol was metabolized to form metabolites such as glucuronidated conjugates. The conjugates are the major forms to be absorbed in the small intestine demonstrated in a Caco-2 cell model via passive diffusion and SGLT-1 pathways similar to quercetin (Henry, Vitrac et al. 2005). It was suspected that resveratrol conjugates might be the substrates for MRP2 which can be effluxed back to the intestinal lumen (Brown, Kroon et al. 2009). More studies are needed to obtain better understanding.

2.5.2 Metabolism

After absorption, most flavonoids and stilbenoids undergo chemical changes following the xenobiotic metabolism. The structure modifications involve three major conjugation pathways also known as Phase II xenobiotic metabolism in the small intestine and in the liver. Specifically, glucuronidation, sulfation and methylation through the actions of uridine- 5'-diphosphate glucuronosyltransferases (UGTs), catechol-O- methyltransferase (COMT), sulfotransferases (SULT). Flavonoids undergo these metabolism pathways to become metabolites which circulate in the body and deposit in the tissue. These metabolites, and not the native plant derived forms, for the most part, are present in tissues and may play major roles in promotion of health and protection against chronic diseases in the body and particularly in the brain.

After being absorbed into the enterocytes, flavan-3-ol monomers undergo metabolism. Small intestine is the main site for glucuronidation catalyzed by UGTs with UGT1 being the major player in flavonoid glucuronidation process (Vaidyanathan and Walle 2002). After first pass through small intestinal metabolism, formed conjugates circulate to the liver via portal vein for further metabolism including methylation by COMT, sulfation by SULT and glucuronidation. Generally, the hydroxyl groups at 3' and 4' position at B ring and 5 and 7 position at A ring are the target for conjugation. It has been identified in studies that flavan-3-ol monomers can form 5-, 7- and 3'-O-glucuronide, 7-O-sulfate, 3'- and 4'-O-methyl, or 3'-O-methyl-7-O-glucuronide, 4'-O-methyl-5- or -7-O-glucuronide (Baba, Osakabe et al. 2000; Natsume, Osakabe et al. 2003;

Spencer, Abd-el-Mohsen et al. 2004; Schroeter, Heiss et al. 2006). Quercetin-glucuronides were found in an in situ model using small intestinal tissue from rats (Crespy, Morand et al. 1999) and also in plasma and urine from humans (Meng, Maliakal et al. 2004). Quercetin sulfates were also found in the in situ model from rat intestine (Crespy, Morand et al. 1999) and in liver microsomes (de Santi, Pietrabissa et al. 2000) with the involvement of sulfotransfearses (De Santi, Pietrabissa et al. 2000). The proposed mechanism on the formation of quercetin glucuronides is that quercetin glycoside is first hydrolyzed to aglycones and then further metabolized to glucuronides by UGTs (Radominska-Pandya, Little et al. 1998; Aumont, Krisa et al. 2001). UGTs has been shown to present in the liver, kidney and intestine in humans which explains the rapid glucuronidation process of polyphenols (Abid, Bouchon et al. 1995; Mojarrabi and Mackenzie 1998; de Santi, Pietrabissa et al. 2000). Methylated quercetin has been found in rat plasma and urine after oral consumption of quercetin (Manach, Morand et al. 1995) catalyzed by COMT. Studies found that peonidin monoglucuronide, cyanidin-3-glucoside, cyanidin monoglucuronide and pelargondin monoglucuronide in human urine after consumption of elderberry or strawberry (Wu, Cao et al. 2002; Felgines, Talavera et al. 2003). Anthocyanin sulfates were also identified in the same study (Wu, Cao et al. 2002; Felgines, Talavera et al. 2003) and SULTs are thought to be the key enzymes responsible for generating the sulfate metabolites. Two possible mechanisms of the formation of anthocyanin glucuronide were proposed to be: 1) anthocyanin glycosides are directly converted to monoglucuronide by UDP-glucose dehydrogenase or are first

hydrolyzed to aglycons and then glucuronidated (Wu, Pittman et al. 2005). Although not reported in plasma, methylated anthocyanins are detected in rat livers (Miyazawa, Nakagawa et al. 1999). Resveratrol glucuronides were identified in human plasma after oral consumption of resveratrol aglycones (Burkon and Somoza 2008). Resveratrol glucuronides were proposed to be generated under similar pathway of quercetin glucuronides which involves aglycones as intermediates to be further glucuronidated (Aumont, Krisa et al. 2001). Resveratrol mono- and disulfates were found in human plasma after oral consumption of resveratrol (Walle, Hsieh et al. 2004; Boocock, Patel et al. 2007). SULTs are thought to be responsible for the sulfation of polyphenol during xenobiotic metabolism (De Santi, Pietrabissa et al. 2000). There is no evidence showing the presence of methylated resveratrol due to the lack of catechol functional group in resveratrol structure and therefore disqualified resveratrol to be a substrate for COMT.

2.5.3 Excretion

After absorption and metabolism, polyphenols reach kidney for further conjugation and are excreted in the urine. Quercetin was reported to have urinary recovery of 0.3% and 1% in men (Hollman, van Trijp et al. 1997). Typically, anthocyanins have very small percentage being excreted in the urine in human, about 0.05% to 0.1% (Wu, Cao et al. 2002; Kay, Mazza et al. 2004; Kay, Mazza et al. 2005). Resveratrol was reported to have relatively high urinary recovery of 26% and 52% in humans (Meng, Maliakal et al. 2004).

The most appropriate approach to estimate polyphenols absorption is to measure their bioavailability which is defined as the percentage of a flavonoid that is absorbed into the bloodstream and available to exert its effect at the target tissue (Ferruzzi 2013). Bioavailability is measured via a pharmacokinetic study designed to determined plasma concentration of a flavonoid as a function of time which describe the onset, duration and intensity of a flavonoid's effect (Clark 1986). Pharmacokinetic parameters including the maximum plasma concentration (C_{max}), time at maximum plasma concentration (T_{max}) and total plasma concentration over a period of time ($AUC_{0\text{-}max}$) are most commonly used to represent the absorption pattern of a flavonoid (Clark 1986). There have been many studies investigating bioavailability of pure flavonoid compounds or flavonoid-rich food on human or animal models. Table 2.5. provides an summary of bioavailability studies conducted on flavan-3-ols, quercetin, resveratrol and anthocyanins either in foods or pure compounds.

Table 2.5. Summary of selected bioavailability studies on flavan-3-ols, quercetin, resveratrol and anthocyanins.

Flavan-3-ol	Model	Formulation	Dose	Duration	Treatment	Target Compound	AUC (nmol*h/L)	Cmax (nmol/L)	Tmax (h)	Extraction method
Goldberg et al. (2003)	human	catechin in v-8, wine or juice	25mg/70kg bw	acute	oral	catechin (free and conjugated)	v-8: 0.26 (0-4h) wine: 0.14 (0-4h) juice: 1.39 (0-4h)	0.14 0.13 0.49	0.5	enzymatic
Abd El Mohsen et al. (2002)	Wistar rats	EC aglycon	100mg/kg bw	acute	gavage	EC 3'-O-MeC EC glucuronide 3'-O-MeC glucuronide 4'-O-MeC glucuronide	4500 1220 44640 14400 1160	blood taken 2h after sacrifice one time point only	2	emzymatic
Baba et al. (2001)	SD rats	EC aglycon	172µmol/kg bw	acute	gavage	EC EC glucuronide EC sulfate EC sulfoglucuronide methyl EC methyl EC glucuronide methyl EC sulfate methyl EC sulfoglucuronide	6480 (0-5h) 39900 (0-5h) 0 (0-5h) 41900 (0-5h) 870 (0-5h) 14900 (0-5h) 41200 (0-5h) 22100 (0-5h)	na	1	emzymatic
Bell et al. (2000)	human	red wine (120mL)	35mg catechin	acute	oral	Total (methylated, unmethylated and sulfated)	371 (0-8h)	78	1.5	emzymatic
Harada et al. (1999)	Wistar rats	EC aglycon	100mg/kg bw	acute	gavage	EC EC glucuronide 3'-O-MeC	2610 (0-6h) 37320 (0-6h) 12.24 (0-6h)	1090 15560 3760	2	emzymatic
Donovan et al. (1999)	human	red wine (120mL)	35mg catechin	acute	oral	Total (methylated, unmethylated and sulfated)	382 (0-8h)	75	1.5	emzymatic
Da Silva et al. (1998)	Wistar rats	EC aglycon	50mg/200g bw	acute	gavage	1h, 6h EC EC sulfate EC glucuronide EC sulfoglucuronide methyl EC methyl EC sulfate methyl EC glucuronide methyl EC sulfoglucuronide	15900 (0-6h) 3600 (0-6h) 64000 (0-6h) 5400 (0-6h) 1200 (0-6h) 16900 (0-6h) 17400 (0-6h) 51900 (0-6h)	13.3 (1h) 3.6 (6h) 52.1 (1h) 5.4 (6h)	1 or 6	emzymatic

Enzymatic extraction method indicates that the samples are treated with β-glucuronidase/sulfatase to deconjugate all polyphenols into free (aglycone) forms.

Table 2.5. Continued.

Quercetin	Model	Formulation	Dose	Duration	Treatment	Target Compound	AUC	Cmax	Tmax	Extraction method
Goldberg et al. (2003)	human	quercetein in v-8, wine or juice	10mg/70kg bw	acute	oral	quercetin (free and conjugated)	v-8: 0.53 (0-4h) wine: 0.88 (0-4h) juice: 0.0033 (0-4h)	0.21 0.42 0.16	0.5	enzymatic
DuPont et al. (2002)	human	apple cider	1.6mg quercetin eq.	acute	oral	3'-methyl quercetinn	na (0-24h)	140	na	enzymatic
Graefe et al. (2001)	human	onions	100mg quercetin eq.	acute	oral	quercetin (free and conjugated)	32.1 (0-24h)	7.6	0.68	enzymatic
Graefe et al. (2001)	human	quercetin 4'-glucoside	100mg quercetin eq.	acute	oral	quercetin (free and conjugated)	27.8 (0-24h)	7	0.7	enzymatic
Graefe et al. (2001)	human	buckweat tea	200mg quercetin eq.	acute	oral	quercetin (free and conjugated)	12.6 (0-24h)	2.1	4.3	enzymatic
Graefe et al. (2001)	human	pure rutin	200mg quercetin eq.	acute	oral	quercetin (free and conjugated)	8.3 (0-24h)	1.1	7	enzymatic
Hollman et al. (1997)	human	apple	107mg quercetin eq.	acute	oral	quercetin (free and conjugated)	3500 (0-36h)	300	2.5	enzymatic
Hollman et al. (1997)	human	onions	68mg quercetin eq.	acute	oral	quercetin (free and conjugated)	7700 (0-36h)	740	0.7	enzymatic
Hollman et al. (1997)	human	pure rutin	100mg quercetin eq.	acute	oral	quercetin (free and conjugated)	3.3 (0-36h)	0.3	9.3	enzymatic

Resveratrol	Model	Formulation	Dose	Duration	Treatment	Target Compound	AUC	Cmax	Tmax	Extraction method
Chow et al. (2010)	human	trans-resveratrol	1g resveratrol	acute	oral	resveratrol res-sulfoglucuronide res monoglucuronide 1 res monoglucuronide 2 res disulfate res-3-sulfate	na	72.2 339.6 619.5 767 359.3 2376.6	only 1h time point	ot mentione
Almeida et al. (2009)	human	trans-resveratrol	150mg, 6x/day	day1 day13	oral	trans-res	140 346	109 280	0.8-1.5	SPE
Boocock et al. (2007)	human	trans-resveratrol	5g resveratrol	acute	oral	resveratrol res-glucuronide 1 res-glucuronide 2 res-3-sulfate	5785 43521 37916 135517	2359 5635 7609 18833	1.5 2 2.5 2.05	SPE
Goldberg et al. (2003)	human	trans-res in v-8, wine or juice	25mg/70kg bw	acute	oral	resveratrol	v-8: 0.073 (0-4h) wine: 0.076 (0-4h) juice: 0.078 (0-4h)	0.037 0.031 0.035	0.5	enzymatic
Biasutto et al. (2002)	rat	trans-resveratrol	0.22mmol/kg bw	acute	gavage	resveratrol res-sulfate res-glucuronide res-disulfate	1100 2400 35500 14400	1200	1	SPE
Marier et al. (2002)	SD rats	trans-resveratrol	50mg/kg bw	acute	gavage	resveratrol res-glucuronide	7000 (0-12) 321900 (0-12)	6570 105000	0.29 0.42	enzymatic

Table 2.5. Continued.

Anthocyanidins	Model	Formulation	Dose	Duration	Treatment	Target Compound	AUC	Cmax	Tmax	Extraction method
Neilson et al. (2003)	human	black currant juice	19.7 mg total antho/kg bw	acute	oral	total ac	19 (0-4)	32-107	0.75	SPE
Neilson et al. (2003)	rabbit	black currant juice	117mg total antho/kg bw	acute	gavage	total ac	13.4 (0-4)	2	0.5	SPE
Rechner et al. (2002)	human	black currant juice (330 mL)	1g total antho	acute	oral	Dp-3-glucoside Dp-3-rutinoside Cy-3-glucoside Cy-3-rutinoside	7.2 (0-6) 40.8 (0-6) 4.1 (0-6) 40.9 (0-6)	3.1-51	1	enzymatic
Matsumoto et al. (2001)	human	black currant extract	3.58mg total antho/kg bw	acute	oral	Dp-3-rutinoside Cy-3-rutinoside Dp-3-glucoside Cy-3-glucoside	287.9 (0-8) 167.6 (0-8) 68.8 (0-8) 9.1 (0-8)	73.4 46.3 22.7 5	1.75 1.5 1.5 1.25	SPE
Matsumoto et al. (2001)	Wistar rats	black currant extract	800 µmol/kg of bw	acute	gavage	Dp-3-rutinoside Cy-3-rutinoside Cy-3-glucoside	1330 (0-8) 2540 (0-8) 1510 (0-8)	580 850 840	2 0.5 0.5	SPE
Cao et al. (2001)	human	elderberry juice (spray-dried)	720mg total antho/kg bw	acute	oral	total ac	na	97	1.1-1.2	SPE
Mazza et al. (2001)	human	blueberry (freeze-dried)	1.2g total antho	acute	oral	total ac	41.4 (0-4)	13.09	4	SPE
Bub et al. (2001)	human	red wine (500 mL)	68mg Mv-3-glucoside	acute	oral	Mv-3-glucoside	288	1.38	0.83	enzymatic
Bub et al. (2001)	human	red grape juice	117mg Mv-3-glucoside	acute	oral	Mv-3-glucoside	662	3	1.5	enzymatic
Miyazawa et al. (1997)	human	red fruit extract (1.6g)	2.7mg Cy-3-glucoside/kg bw	acute	oral	Cy-glucoside	na	29	1	SPE
Miyazawa et al. (1997)	SD rats	red fruit extract	320mg Cy-3-glucoside/kg bw	acute	gavage	Cy-glucoside	3.6 (0-4)	7.8	0.25	SPE

2.6 Tissue Distribution of Polyphenols

Low bioaccessibility and bioavailability in gastrointestinal tract (GI) leave flavonoid metabolites to circulate in low concentrations ranging from μM to nM depending on the dose and the specific nature of the phenolics. Level of tissue distribution is even less well known for flavonoids after GI absorption and excretion. Data on storage tissue has been identified for flavonoid metabolites and their presence in tissues is considered to be transient (El Mohsen, Marks et al. 2004; Tsang, Auger et al. 2005; El Mohsen, Marks et al. 2006; Juan, Maijo et al. 2010). However, the general notion remains that these bioactive flavonoids must reach target tissues to provide specific protective benefits. This requirement poses a hurdle in the case of neuroprotective polyphenols due to the difficulties in crossing the blood-brain barrier.

2.6.1 Blood-Brain Barrier and Efflux Transporters

The blood-brain barrier is a structure consisted of endothelial cells to form the walls of capillaries. The tight junctions between the endothelial cells regulate the exchange of nutrients and function as a protective barrier for toxins (Abbott, Patabendige et al. 2010). Polyphenols are considered xenobiotics and undergo phase I and II xenobiotic metabolism as described previously. Their xenobiotic properties make them substrates to many efflux transporters responsible for gate-keeping at the blood-brain barrier. The most studied efflux transporters are ATP-binding cassette transporters which include p-glycoprotein, multidrug resistance-associated proteins and breast cancer resistance protein. Youdim et al. conducted a series of studies to investigate flavonoid permeability across blood-brain barrier using *in*

vitro (ECV/304C6 coculture) cell model. They demonstrated that flavonoid permeability dependent on their lipophilicity and polarity. Compounds are less polar (i.e. O-methylated conjugates) have better permeability than more polar ones (i.e. sulfated and glucuronidated conjugates) (Youdim, Dobbie et al. 2003). They further demonstrated that flavonoids are the substrates of p-glycoprotein and the permeability dependent on their interaction with efflux transporters (Youdim, Qaiser et al. 2004). More recently, Faria et al. demonstrated that stereoselectivity (epicatechin >> catechin) is also a determining factor for brain permeability (Faria, Pestana et al. 2011). These *in vitro* studies sparks the interests in more *in vivo* studies investigating brain concentrations of polyphenols to support their neuroprotective properties. However, to date only a few studies have documented the presence of polyphenols in brain tissues.

2.6.2 Current Evidence of Polyphenol Deposition in Brain Tissue

In order to fill the gap of knowledge on brain accumulation of polyphenols, a summary of current data is generated in Table 2.6. to illustrate the knowledge up to date including the basic parameters on the study design (species, dose, formulation and etc.). Based on what we have known here, our research was designed to fill the gap of what is unknown including the effects of diet composition (Chapter 3), disease condition on polyphenol brain deposition (Chapter 4) and also the regional brain deposition differences of polyphenols (Chapter 5).

Table 2.6. Summary of current data on favan-3-ols, quercetin, resveratrol and anthocyanin deposition in brain tissues.

Flavan-3-ol	Model	Formulation	Dose	Duration	Treatment	Harvest Approach	Time at harvest	Target Compound	Brain Conc.	unit	Extraction
Serra et al. (2012)	Wistar rats	procyanidin extarct (PE)	50mg PE	acute	oral	NA	4h	MeC-sulfate	6.4	nmol/g, 4h	SPE
Ferruzzi et al. (2009)	SD rats	grape seed extract	50-100mg/kg bw dose escalation	10d	gavage	perfusion	1h	C+3'OMeC EC+3'OMeEC	290.7 576.7	pg/g	enzymatic
Prasain et al. (2009)	SD rats	grape seed extract	300 mg/kg bw	2x/day for 3d	gavage	perfusion	4h	C EC	53.16 NA	ng/g	enzymatic
Chu et al. (2007)	SD Dam -> fetal organs	green tea extract	550 mg/kg bw	acute at 15.5d of gestation	gavage	rinse	C: Tmax=0.5 h EC: Tmax=0.5 h	C EC	Cmax=0.7 Cmax=38.9	pmol/g	SPE
Abd El Mohsen (2002)	Wistar rats	EC aglycon	100mg/kg bw	1d	gavage	perfusion	immediately	EC glucur 3'Me-EC glucur	<LOQ	NA	enzymatic
Suganuma et al. (1998)	CD-1 mice	(3H)EGCG	0.05%	actue	gavage	NA	NA	(3H)EGCG	$17.3X10^3$	dpm/g	NA
Nakagawa and Miyazawa (1997)	SD rats	EGCG	500mg/kg bw	acute	gavage	perfusion	1h	EGCG	0.5	nmol/g	liquid

Quercetin	Model	Formulation	Dose	Duration	Treatment	Harvest Approach	Time at harvest	Target Compound	Brain Conc.	unit	Extraction
Ho et al. (2013)	SD rats	Cabernet sauvignon	150 mg tp/kg bw	10d	gavage	perfusion	1h	Q-3-O-glucur Me-Q-glucur	0.91 NQ	pmol/g	SPE
Ishisaka et al. (2011)	Wistar rats	Q aglycon	1%	1 mo	diet	perfusion	1h	Q aglycon MeQ	40.1 47.7	pmol/g	enzymatic
Rangel-Ordonez et al. (2010)	SD rats	Ginkgo biloba	600mg/kg bw 600 mg/kg bw	acute 8d	gavage	rinse	immediately	actue:Q aglycon acute:MeQ 8 d:2x increae in MeQ	nd 161	ng/g	enzymatic
Huebbe et al. (2010)	C57BL/6J mice	Q aglycon	2g/kg diet	6wks	diet	NA	NA	Q aglycon MeQ	0.28 0.08	nmol/g	enzymatic
Bierger et al. (2008)	pig	Q aglycon	50mg/kg bw	4 wks	diet	NA	1-1.5h	Q aglycon	0.02	nmol/g	enzymatic
de Boer et al. (2005)	Fisher 344 rat	Q aglycon	1% (500-800 mg/kg bw)	11wks	diet	NA	NA	Q aglycon	0.06	nmol/g	enzymatic
de Boer et al. (2005)	pig	Q aglycon	500mg/kg bw	3d	diet	NA	NA	Q aglycon	0.22	nmol/g	enzymatic
Mullen et al. (2002)	vatt Hooded Lister	[2-14C]Q-4'-glucoside	$58.5x10^5$ dpm	acute	with feed	perfusion	1h	[2-14C]Q-4'-glucoside	0	)3/g/ml fresh	NA

NA: not available.

SPE: Solid phase extraction

Table 2.6. Continued.

Resveratrol	Model	Formulation	Dose	Duration	Treatment	Harvest Approach	Time at harvest	Target Compound	Brain Conc.	unit	Extraction
Lin et al. (2012)	SD rats	Polygonaceae	4g/kg bw	2x/d, 7 does	gavage	perfusion	30min	Res glucur Res sulafte	ND	NA	enzymatic
Juan et al. (2010)	SD rats	trans res	15mg/kg bw	acute	i.v.	NA	30min	trans-res res-glucur res-sulfate	0.17 ND 0.04	nmol/g	liquid
Vingtdeux et al. (2010)	SD rats	trans res	100-400 mg/kg bw dose escalation	3 days	gavage	perfusion	1h	res aglycon	1.7	nmol/g	SPE
Abd El Mohsen et al. (2006)	SD rats	[3H]trans-resveratrol	50mg/kg bw 1.85 Mba(3H)trans-res	?	gavage	acute	2 or 18h	(3H)trans-res	2h:0.03% of oral dose 18h:0.01% of oral dose	NA	NA

Anthocyanidins	Model	Formulation	Dose	Duration	Treatment	Harvest Approach	Time at harvest	Target Compound	Brain Conc.	unit	Extraction
Ho et al. (2013)	SD rats	Cabernet sauvignon	150 mg tp/kg bw	10d	gavage	perfusion	1h	Mv-glucoside Mv-glucopyruvate Mv-glucur	12.3 ± 3.85 6.4 ± 5.2 ND	pmol/g	SPE
Del Bo et al. (2010)	SD rats	blueberry	8% in diet	4 or 8wks	diet	perfusion	NA	native forms	ND	NA	SPE
Milbury and Kalt (2010)	pigs	blueberry	2% in diet	8wks	diet	rinse only	0.5h	native forms and glucur	cortex:0.7 midbrain:0.92 cerebellum:0.87	nmol/kg	SPE
Kalt et al. (2008)	pigs	blueberry	1 or 2 or 4%	4wks	diet	rinse only	0.5h	native forms	detected in cortex and cerebellum only%	NA	SPE
Abd El Mohsen et al. (2006)	SD rats	pelargonidin	50 mg/kg bw	acute	gavage	perfusion	2h	pelargonidin aglycon	0.16	nmol/g	SPE
Andres-Lacueva et al. (2005)	F334 rats	blueberry	2%	10wks	diet	blood free	NA	native forms	ND in cortex hippocampus cerebellumn striatum	NA	SPE
Talavera et al. (2005)	Wistar rat	blackberry	15g/kg bw	15d	diet	perfusion	3h	cy-3-glc	0.21 + 0.05	nmol/g	SPE
Passamonti et al. (2005)	Wistar rat	red grape extract	8mg/kg bw	acute	gavage	rinse	10min	native forms	192.2 + 57.5	ng/g	SPE

2.7 Objectives and Hypotheses

While the neuroprotective benefits of polyphenols are promising, a key step in understanding the potential of these plant derived compounds to promote healthy brain development and neurodegenerative processes is the determination of dose response, bioavailability, metabolism and brain tissue distribution of specific polyphenol forms. However, limited data exists on 1) brain accumulation concentrations of polyphenols, 2) the estimation of polyphenol intake on infants and children and 3) factors that influence polyphenol bioavailability. The following hypotheses are formulated to provide better understanding regarding these questions.

Hypothesis I: Diabetogenic diet and diabetic condition will influence polyphenol absorption and brain deposition.

Specific aim 1A: To investigate the impact of diet composition especially the diabetogenic diet high in fat on central and peripheral polyphenol bioavailability in a Sprague-Dawley rat model.

Specific aim 1B: To investigate the effects of diabetic condition on central and peripheral polyphenol bioavailability in a Zucker diabetic fatty rat model.

Since dietary pattern has been shown to influence the susceptibility to neurodegenerative diseases. It is important to understand the effect of dietary fat especially diabetogenic diet on brain and plasma polyphenol bioavailability in order to better connect the risk of neurodegeneration with dietary pattern. Follow on that concept, chronic diabetogenic fat increase risk to type II diabetes. In order to study how

diabetogenic diet and disease condition impacts on polyphenol absorption, Sprague-Dawley and Zucker diabetic rats models will be used to carried out the studies.

Hypothesis II: Polyphenols metabolites will distributed differentially in brain regions.

Specific aim 2: To explore the regional brain bioavailability of polyphenols from apple/GSE and bilberry extract in a young swine model.

There is no known data on regional deposition of polyphenols in brains. This aim seeks to determine the regional differences on key polyphenol metabolites from apple/GSE and bilberry extract at a pharmacological (82.5 mg/kg body weight) or physiological (27.5 mg/kg body weight) dose in pig brain. We hypothesize that polyphenol metabolites will distributed differentially in brain regions.

CHAPTER 3. PLASMA BIOAVAILABILITY AND BRAIN DISTRIBUTION OF GRAPE POLYPHENOLS IN SPRAGUE-DAWLEY RATS FED ON HIGH FAT DIET

As a part of manuscript titled 'Role of Standardized Grape Polyphenol Preparation as a novel treatment to improve synaptic plasticity through attenuation of features of metabolic syndrome' submitted to Nutrition & Metabolism

Abstract

Consumption of grape derived polyphenols is associated with the prevention of neurodegenerative diseases such as Alzheimer's disease (AD). High fat diet (HF), on the other hand, has been shown to be a risk factor on AD onset and progression. To further understand the physiological impacts of polyphenol-rich compounds ingested as a grape polyphenolic preparation termed 'Standardized Grape Polyphenol' (SGP) and the influence of dietary fat on absorption and metabolism of flavan-3-ols, flavonols and anthocyanins, studies were conducted with Sprague-Dawley (SD) rats fed on HF (60%kcal%fat) or control diet (10%kcal%fat). Rats were randomly assigned to four groups: control (CNTL) diet-water, HF diet-water, CNTL diet-SGP, HF diet-SGP. Animals were fed on individual diet for 2 weeks prior to water or SGP treatment. Plasma pharmacokinetics was determined after SGP or water dosing for 10 days. LC-MS/MS analyses confirmed the detection of catechin-5-glucuronide (C-5-glucur), epicatechin-5-

glucuronide (EC-5-glucur), 3'-O-methylcatechin-5-glucuronide (3'-OMeC-5-glucur), 3'-O-methylepicatechin-5-glucuronide (3'-OMeEC-5-glucur), quercetin-3-glucuronide (Q-3-glucur), methylquercetin-glucuronide (MeO-Q-glucur) and resveratrol-3-glucuronide (Res-3-glucur) to be the main metabolites in plasma and brains from repeated SGP treatment regardless the diet difference. Anthocyanins were detected in plasma and brains following 10 days of SGP treatment regardless fat content in the diet. These data illustrate that dietary fat content had minimal influences on plasma bioavailability of polyphenols from SGP. SGP polyphenol access to the brain was also not influenced by fat content in the diet.

3.1 Introduction

Excessive fat consumption has become a major concern in the United States and worldwide. HF consumption has been associated with high incidence of obesity, dyslipidemia and other systemic metabolic dysfunction that were defined as metabolic syndromes (Scarpellini and Tack 2012). According to the dietary reference intake (DRI) report on macronutrient published by National Academy of Science in 2002, the adequate intake (AI) of total fat is recommended as 30 g/d for all age groups (IOM 2002). However, the NHANES data reported that during the period of 2009-2010, the mean amount of total fat consumed by adult Americans was 93.3 g/men and 66.0 g/women (U.S. Department of Agriculture 2012). This data suggests that U.S. adults consume about 2 to 3 times over the recommended fat amount. Habitual HF intake is therefore a

key contributor to obesity which extends to metabolic syndromes and more severely, complications of type II diabetes. Furthermore, epidemiological studies have demonstrated a clear link between obesity and neuro-cognitive disorders. For example, people with obesity possessed a four times higher risk for AD (Pasinetti, Wang et al. 2011). In fact, HF consumption as a precursor of metabolic syndromes may be considered a factor that places individuals at risk for AD. It is evident that type II diabetes is associated with high risk to AD (Ott, Stolk et al. 1996; Stolk, Breteler et al. 1997; Craft and Watson 2004). It has been shown in Tg2576 mice, a mouse model of AD, the diabetogenic diet composed of 60% fat as lard that the diet promoted β-amyloid plaque burden in the brain of the AD mice (Pasinetti, Wang et al. 2011). This result suggested that HF diet promotes AD but raised another question as to how the HF influences absorption and metabolism of protective polyphenols from the diet. Our interest in development of dietary AD preventative strategies based on grape polyphenols, it is critical to understand the impacts of HF diet on grape polyphenol absorption and metabolism. These data will provide critical information in determining the dosage for future efficacy testing on habitual HF consuming population who also are at high risk of AD.

We have previously demonstrated the bioavailability and brain distribution of Q metabolites resveratrol and anthocyanins from SD rats repeatedly treated with Concord grape juice (unpublished), resveratrol (Marambaud, Zhao et al. 2005) and GSE (Wang, Ho et al. 2008; Ferruzzi, Lobo et al. 2009) individually. Efficacy evidence on individual

GSE and resveratrol on ameliorating AD have been demonstrated *in vitro* and *in vivo* in a AD mouse model (Marambaud, Zhao et al. 2005; Wang, Ho et al. 2008; Wang, Thomas et al. 2009). Considering these products provide distinctly different polyphenol classes (GSE – flaval-3-ols; Concord grape juice – anthocyanins and quercetin; Stilbenoid – resveratrol) that each target different molecular mechanism related to AD. There is interest in devising combination strategies favoring delivery of these polyphenols to brain tissues. However, combination treatments can alter absorption and metabolism of individual polyphenols. We, therefore, propose to investigate the absorption profile and tissue distribution of polyphenols from a combination of grape products termed SGP using a SD rat model. Given the close relationship between HF consumption and AD, the effect of HF diet on SGP plasma bioavailability and brain distribution was investigated.

3.2 Materials and Method

Chemicals and Materials.

(+)-C, (-)-EC, Q-3-glucoside, Q-3-glucur and Q aglycon standards were purchased from Sigma Chemical Co. (St. Louis, MO). All extraction and LC solvents were HPLC certified and were obtained from J.T. Baker (Phillipsburg, NJ). GSE Meganatural® AZ powder was from Polyphenolics (Madera, CA). 100% Concord grape juice (Welch's) was loaded onto C18 Solid Phase extraction cartridges (1 c.c.) and washed with ddH_2O (0.01% HCl) to remove sugar. Polyphenols were then eluted with MeOH (0.01% HCl). MeOH was then removed under vacuum and resulting concentrate was kept frozen until use. trans-

resveratrol, malvidin (Mv)-3-glucoside chloride and cyanidin (Cy)-3-glucoside chloride were purchased from ChromaDex (Irvine, CA).

Animal and Diet.

Animal studies were conducted under guidance and with protocols approved by the Purdue University Animal Care and Use Committee. Bioavailability of SGP polyphenols including anthocyanins, monomeric flavan-3-ols and Q were assessed in a male SD rat model as described by Ferruzzi et al. (Ferruzzi, Lobo et al. 2009). Briefly, sixteen SD rats weighed between 275 and 300g were purchased from Harlan Inc. (Indianapolis, IN). Rats were randomly assigned to CNTL diet-water, HF diet-water, CNTL diet-SGP or HF diet-SGP groups for two weeks prior to initiation of water or SGP treatment. All animals were given deionized water ad libitum. HF diet (D12492) containing 60% kcal% fat from lard and CNTL diet (D06041501P) containing 10% kcal% fat were purchased from Research Diets Inc. (New Brunswick, NJ). The diet composition is listed in Table 3.1. Body weight and food intake were monitored every other day throughout the study. According to the food intake, caloric intake was calculated based on the energy the diets provided. HF diet provided 5.24 kcal/g pellet and CNTL diet provided 3.85 kcal/g pellet. After two weeks on CNTL or HF diet, rats were treated with deionized water or SGP by daily intragastric gavage for ten days. SGP consisted of 150 mg/kg BW of GSE, 62 mg/kg BW of Concord grape juice and 297 mg/kg BW of trans-resveratrol all based on the total phenolic contents. Total polyphenol contents for GSE is 58% wt/wt, resveratrol is 69%

wt/wt and Concord grape juice is 0.23% wt/v analyzed by Folin-Ciocalteu assay. Appropriate amount of SGP was mixed with deionized water to 1.0 mL and delivered to rats as the first gavage. Residual doses were rinsed with 0.5 mL deionized water and administered to rats as the second gavage. Rats were gavaged twice a day, 8h apart to reach the proper dosage.

Table 3.1. Diet composition of HF and CNTL diet.

Ingredient	Kcal % HF	Kcal % CNTL
Protein	20	20
Carbohydrate	20	70
Fat	60	10
Total	100	100
Kcal/gm	5.24	3.85

Other Ingredients	kcal	kcal
Casein	800	800
L-Cysteine	12	12
Corn Starch	0	1260
Maltodextrin	500	140
Sucrose	275.2	1400
Cellulose	0	0
Soybean oil	225	225
Lard	2205	180
Mineral mix	0	0
Vitamin mix	40	40
Blue Dye	0	0
Total	**4057**	**4057**

SGP Pharmacokinetics.

The procedure of the pharmacokinetic study was based on (Ferruzzi, Lobo et al. 2009). In brief, at the eighth day of SGP treatment, rats were anesthetized by given 3-5% of isoflurane in the anesthesia chamber and maintained with a mask with 1.5-3% isoflurane. A handmade polyethylene catheter was implanted into a rat's jugular vein

for blood draw. Rats were injected with Buprenex (0.01-0.05mg/kg) before regaining consciousness to alleviate pain. Catheters were kept patent by flushing with heparinized saline (100 units/mL) every 12 h. Rats were allowed to rest for 24 h to recover after surgery. After fasted for 8h, a pharmacokinetic study was initiated and food was offered again 2 h after SGP treatment. Pharmacokinetics was conducted based on the following schedule by collecting 400 µL of blood at 0 (before gavage), 0.25, 0.5, 1, 2, 4, 6, 8 h post gavage from the jugular catheter into heparinized tubes. Heperinized blood was centrifuged at 6500 rpm for 10 min at 4 ℃ resulting in 200 µL of plasma. Plasma was acidified with acidified saline (1% ascorbic acid wt/v) in 4:1 ratio, purged with N_2 and stored at -80 ℃ until analysis. The day after pharmacokinetics, another dose was administered and rats were sacrificed 1h post dose. Rats were then perfused with ice-cold saline to remove possible blood contamination. Brain tissues were harvested and snap frozen in liquid nitrogen.

Polyphenol Metabolites Extraction in Plasma and Brain Tissues.

Monomeric flavan-3-ols, resveratrol and Q metabolites were extracted from plasma (~100 µL) by SPE using 1 cc Waters Oasis HLB cartridges (Milford, MA). Acidified plasma samples were thawed in room temperature, brought up to 0.5 mL with acidified saline (0.1% formic acid v/v) and vortex for 5s. SPE cartridges were preconditioned with 1 mL of ddH_2O followed by 1 mL of methanol and samples were loaded onto the cartridges. The cartridges were then cleaned with 1 mL of 1.5M formic acid (v/v) followed by 1 mL

of 5% methanol (v/v). After cleaning, polyphenols in plasma were eluted with 2 mL of methanol and dried under vacuum at 37 ℃. Dried phenolic extracts were reconstituted with 0.1% aqueous formic acid (v/v) and 0.1% formic acid in acetonitrile (v/v) in 4:1 ratio. Reconstituted samples were vortex and sonicated for 10s and analyzed by LC-MS/MS. Brain tissues were weighed and finely diced into pieces and homogenized with ice-cold methanol (9 mL/g tissue) until a homogenate was obtained. Brain homogenates were centrifuged at 3700 rpm at 4 ℃ for 10 mins. Top layer methanol was collected. The methanol extraction process was repeated again with 6 mL of methanol/g tissue. Methanol was dried under 37℃ vacuum. Dried residuals were reconstituted with 1 mL 0.1% aqueous formic acid (v/v) and underwent the same SPE procedure as plasma described above.

Anthocyanins were extracted using the same Waters Oasis HLB cartridges and the same procedure except for the use of different solvents. SPE cartridges were preconditioned with 3 mL of ddH_2O followed by 3 mL of methanol and samples were loaded onto the cartridges. 3 mL of 2% aqueous formic acid (v/v) was used to wash the cartridges. Anthocyanins were eluted with 2 mL of 2% formic acid in methanol (v/v) and dried under vacuum at 37℃. Dried extracts were resolubilized in 100 µL of 2% aqueous formic acid (v/v) for LC-MS/MS analysis. Anthocyanins in brain tissues were extracted with ice-cold methanol followed the same procedure as above and underwent anthocyanin SPE.

LC-MS Analysis of SGP Material, Plasma and Brain Tissues.

SGP material. SGP containing 61.8 mg resveratrol, 34.0 mg GSE and 0.40 mL Concord grape juice was analyzed LC-MS by Rutgers University. This preparation was adopted from half of the animal dosage based on a rat weighed ~250 g. HPLC separation was performed on a Polaris amide-C18 column, 250 x 4.6mm, 5um (Varian Inc.). For LC-MS analysis, a Hewlett Packard Agilent 1100 Series LC-MS (Agilent Technologies, Waldbronn, Germany) equipped with autosampler, quaternary pump system, DAD detector, degasser, MSD trap with an electrospray ion source (ESI), and software for data processing was HP ChemStation. The identification of anthocyanins, flavonols, phenolic acids and flavanols were achieved by using the same LC gradient, but with different MS ionization mode and mobile modifiers. Anthocyanins and flavonols were detected under positive ion mode with 0.4% TFA (v/v) in water and ACN. Phenolic acids and flavanols/proanthocyanidins were detected under negative ion mode with 0.1% formic acid (v/v) in water and ACN. HPLC separation was performed with the mobile phase containing solvent A (0.4% TFA or 0.1% FA in water) and B (0.4% TFA or 0.1% FA in ACN) in gradient: 0-20 min, linear gradient from 10 % to 20 % B; 20-30 min, linear gradient from 20% to 30% B; 30-40 min, isocratic elution at 30% B; 40-50 min, linear gradient from 30% to 50%; 50-60 min, linear gradient from 50% to 60%. The flow rate was set at 1.0 mL/min. The UV detector was set at 254, 280, 370, 520 nm. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) scanned from *m/z* 100 to 1200. ESI was conducted by using a needle voltage of 3.5 KV (positive) and -3.5 KV

(negative). High purity nitrogen (99.999%) was used as dry gas at a flow rate of 12 L/min and capillary temperature was at 350°C. Nitrogen was used as nebulizer at 60 psi, and Helium as collision gas. For the detection of resveratrol, an isocratic method was used consisting of 60% solvent A (40 μM ammonium acetate in ddH2O) and 40% solvent B (acetonitrile). The flow rate was 0.5mL/min, and the run length was 20 minutes. The UV detector was set at 305nm, and MS detection was set under negative mode. The eluent was monitored by electrospray ion mass spectrometer (ESI-MS) scanned from *m/z* 100 to 600. ESI was conducted by using a needle voltage of -3.5 KV (negative). High purity nitrogen (99.999%) was used as dry gas at a flow rate of 8 L/min and capillary temperature was at 350°C. Nitrogen was used as nebulizer at 40 psi, and Helium as collision gas.

Flavan-3-ol and Q metabolites LC-MS Analyses.

Analyses of flavan-3-ol metabolites from plasma was performed on an Agilent 1100 time of flight. Analysis of all brain samples and plasma for Q metabolites and anthocyanins was performed on an Agilent 6400 Triple Quad equipped with Jetstream electrospray ionization (ESI) source under multiple reaction monitoring modes (MRM). A Waters XTerra RP-C18 column (2.1 mm x 100 mm, 3.5 μm particle size) was used on both systems. A binary mobile phase system consisted of A: 0.1% aqueous formic acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v) was used. The column was heated to 30℃ and the system flow rate was 0.3 mL/min. The binary gradient to elute monomeric

flaval-3-ols and Q metabolites was described as followed: 10% B at 0 min, 40% B at 5.5 min, 70% B at 7 min, 95% B at 7.5 min and back to 10% B at 8.5 min and continue onto 13.5 min. MS data was obtained under negative polarity. ESI source conditions: gas temperature was 350℃, drying gas flow was 11 l/min, nebulizer was 30 psi, sheath gas temp was 350℃, sheath gas flow was 11 L/min, capillary voltage was 3500V and nozzle voltage was 1000V.

Anthocyanin Analysis.

A Waters XTerra RP-C18 column (2.1 mm x 100 mm, 3.5 µm particle size) was used and heat up to 35℃. The binary mobile phases were A: 2% aqueous formic acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v). The LC gradient is 5% B at 0 min, 10% B at 10 min, 25% B at 30 min, 5% B at 31 min and continue on 5% B to 35 min. MS data was obtained under positive polarity. MS condition was the same as described above.

Data Analysis.

Data were expressed as mean ± standard error of mean (SEM). Pharmacokinetic parameters including plasma area under the curve from 0 to 8h ($AUC_{0\text{-}8h}$) was calculated using the trapezoidal rule and the maximum plasma concentration (C_{max}) and the time at the maximum plasma concentration (T_{max}) were obtained from the pharmacokinetic curves expressed as plasma concentration versus time. Significance among groups on body weight gain, food intake and energy intake were determined using one way

ANOVA by SAS 9.3 (Cary, NC) with Tukey's post hoc test to determine pairewise differences. The differences between HF-SGP and CNTL-SGP groups on pharmacokinetic parameters and brain levels were determined by Student's test. Significance was accepted at the level of $\alpha<0.05$.

3.3 Results

SGP Material Profile.

The major flavan-3-ols, flavonols and anthocyanins from SGP were detected by UV at 280, 370 and 520 nm determined by Dr. Jim Simon from Rutgers University. Quantification of major polyphenols in SGP was shown in Table 3.2

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Table 3.2. Major polyphenols in SGP.

Polyphenol Class	Flavonols		Flavan-3-ols		Anthocyanins					Stilbenoids
Polyphenols	Q-3-gluc	Q-3-glucur	C	EC	Mv-gluc	Pt-gluc	Dp-gluc	Pn-gluc	Cy-gluc	Resveratrol
Concentration (mg/100g)	3.5	5.5	546	649	1.4	1.5	3	trace	2.3	11427

Animal body weight and food intake.

Mean body weight gain was calculated by the difference from the day rats started respective diet and the day before jugular vein surgery. After surgery, rats tended to lose weight which might create bias. There was no significant difference among groups on mean body weight gain, however, there was a slightly decrease in HF-water and HF-

SGP groups compared to rats in CNTL diet groups (Figure 3.1.). The decrease in body weight in both HF groups was consistent with the decreased food intake (Figure 3.1.). Despite the decrease in body weight change and food intake, the energy intake for both HF groups was positively increases compared to the CNTL diet groups, however, the trend showed no significance (Figure 3.1.). Our results concluded that repeated dose of SGP did not influence body weigh gain, food intake and energy intake regardless the fat content in diets.

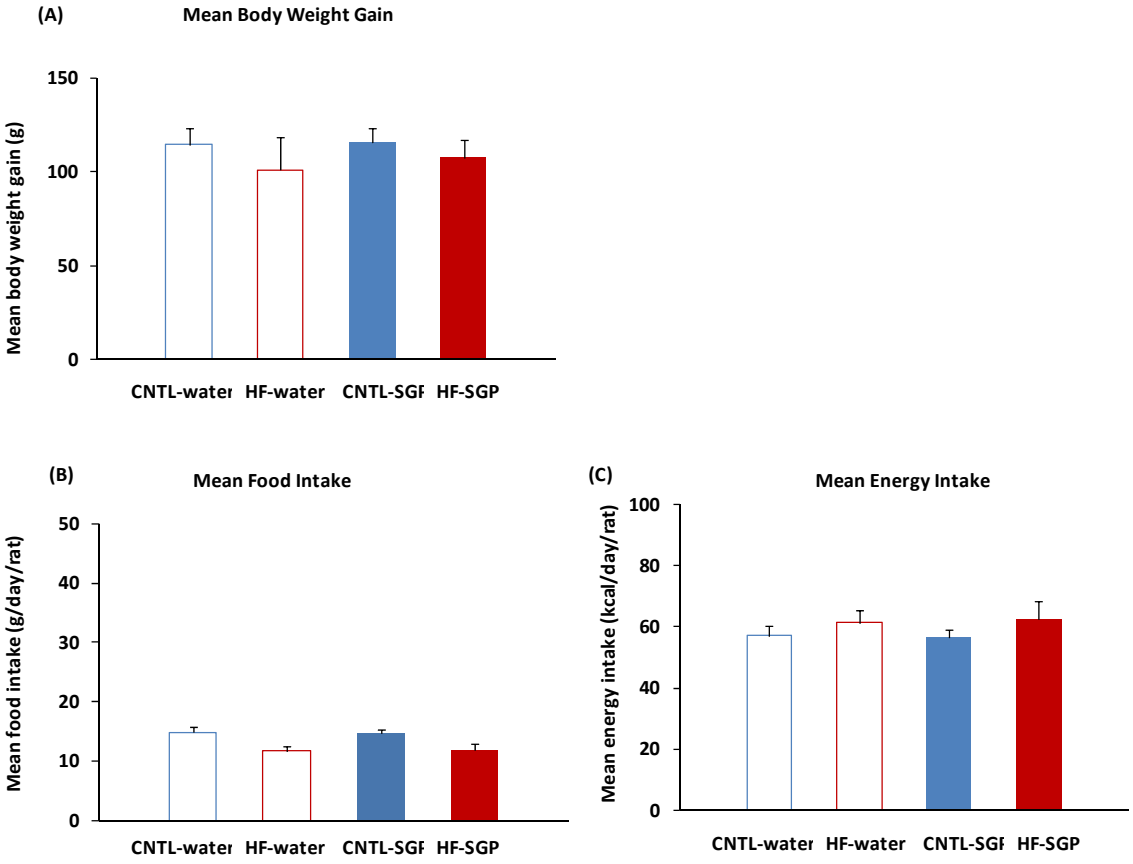


Figure 3.1. Mean body weight change, mean food intake and mean energy intake of the rats.
Data represented as mean ± SEM. (n=4/group).

SGP Bioavailability in SD Rat Plasma.

Flavan-3-ol. Flavan-3-ols, C and EC, from GSE in SGP were metabolized to produce catechin-5-glucuronide (C-5-glucur), epicatechin-5-glucuronide (EC-5-glucur), 3'-O-methylcatechin-5-glucuronide (3'OMeC-5-glucur)and 3'-O-methylepicatechin-5-glucuronide (3'OMeEC-5-glucur) found in plasma and brain tissues. The eight hour pharmacokinetic profiles were depicted in Figure 3.2. All four flavan-3-ol metabolites reached T_{max} within 1 to 2 hour after SGP treatment regardless the background diet condition (Table 3.3.). Rats on CNTL diet showed no significant differences on C_{max} and $AUC_{0\text{-}8h}$ on C-5-glucur, 3'MeOC-5-glucur and 3'MeOEC-5-glucur compared to rats fed on HF diet suggesting that plasma bioavailability was not affected by background diet fat content. One exception was EC-5-glucur in which rats fed on CNTL diet had significantly lower C_{max} ($p=0.02$) and $AUC_{0\text{-}8h}$ ($p=0.03$) compared to rats fed on HF diet.

Quercetin. Quercetin-3-glucuronide (Q-3-glucur) and methylquercetin glucuronide (MeO-Q-glucur) were detected in plasma from rats repeatedly treated with SGP for 10 days. The eight hour pharmacokinetic behavior was shown in Figure 3.3. MeO-Q-3-glucur reached T_{max} at 30 min after SGP treatment for HF and CNTL diet groups. Q-3-glucur peaked between 15 min and 30 min for HF and CNTL diet groups after SGP treatment. Plasma $AUC_{0\text{-}8h}$ was determined at nano molar range. Rats showed similar $AUC_{0\text{-}8h}$ and C_{max} regardless the fat content in diets on Q-3-glucur and MeO-Q-glucur (Table. 3.4.).

Resveratrol. Resveratrol-3-glucuronide (Res-3-glucur) was the main metabolite detected in plasma and brain tissues from resveratrol fraction of SGP. Eight hour pharmacokinetic response of Res-3-glucur are depicted in Figure 3.3. Rats on CNTL diet group showed significantly higher plasma C_{max} ($p=0.02$) compared to the rats on HF diet group suggesting that HF diet might hinder Res-3-glucur bioavailability. However, AUC_{0-8h} was not significantly different between CNTL and HF groups (Table 3.5.).

Anthocyanins. Five major anthocyanins including Mv, Cy, Dp, Pn and Pt-glucoside were detected in plasma and brain tissues. There were no datectable anthocyanidin-glucuronides found in plasma. Pharmacokinetic curves of anthocyanins are shown in Figure 3.4. All anthocyanins demonstrated T_{max} at 0.25h regardless of fat content in diets. This is consistent with other reports that demonstrated the rapid plasma increase in the gastric environment is indicative that stomach is a major absorption site for anthocyanins (Passamonti, Vrhovsek et al. 2003; Talavera, Felgines et al. 2003). There were no significant differences found in C_{max} and AUC_{0-8h} on anthocyanins between CNTL and HF diet groups (Table 3.6.). Although Cy-3-glucoside was the second highest abundant anthocyanin components in the Concord grape juice, it showed the highest C_{max} (9.61 and 8.97 nmol/L plasma for CNTL and HF diet group) compared to the other anthocyanins (average of 4.00 nmol/L plasma). Overall, fat content in the background diet seemed to have no effects on plasma bioavailability of anthocyanins (Table 3.6.).

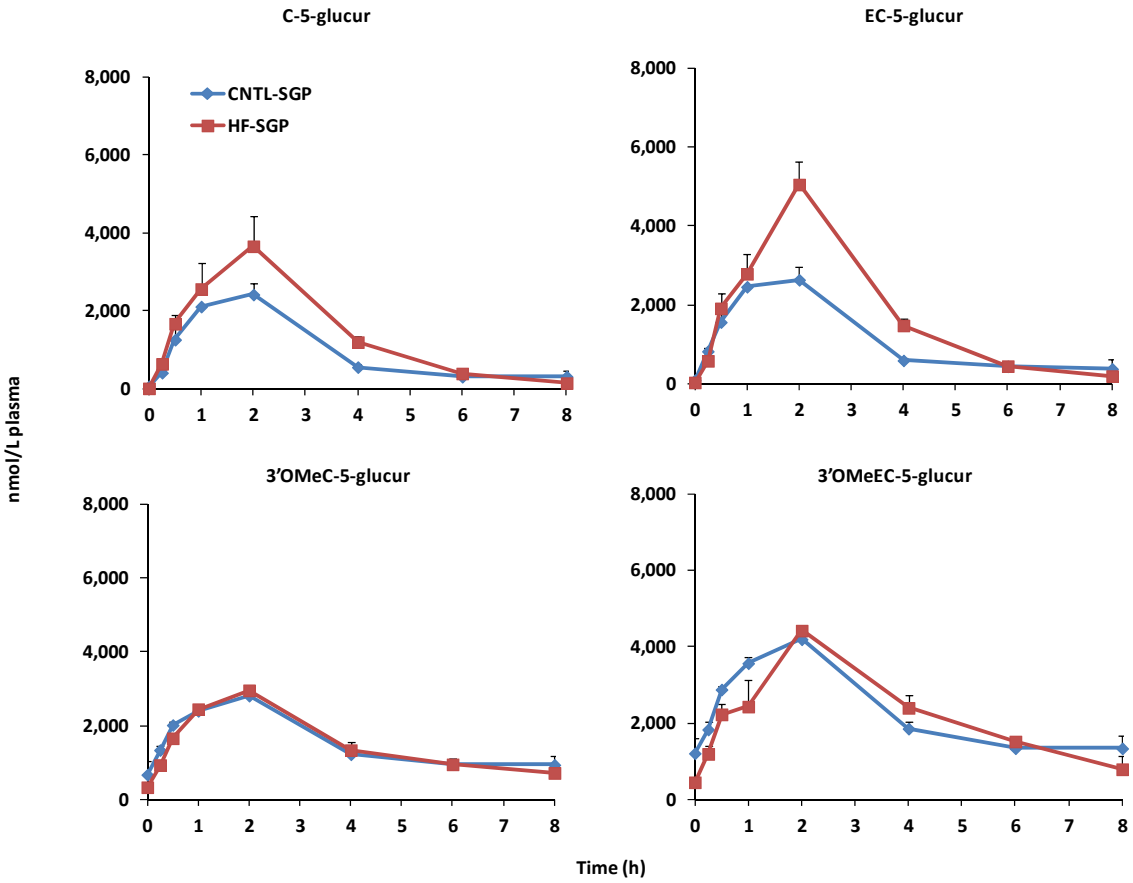


Figure 3.2. Plasma pharmacokinetic responses of flavan-3-ol metabolites.
Data represented as mean ± SEM (n=3/group).

Table 3.3. Pharmacokinetic parameters of flavan-3-ol metabolites.

Diet	Compound	AUC (nmol/L-h)	C_{max} (nmol/L)	T_{max} (h)
CNTL	C-5-glucur	7888.9 ± 186.5	2458.4 ± 263.4	1.7 ± 0.3
HF		9192.9 ± 747.5	3660.6 ± 783.4	2.0 ± 0.0
CNTL	EC-5-glucur	9122.2 ± 303.9	2791.4 ± 188.7	1.7 ± 0.3
HF		14654.0 ± 1571.7*	5058.4 ± 588.6*	2.0 ± 0.0
CNTL	3'OMeC-5-glucur	12566.0 ± 557.4	2820.5 ± 67.8	2.0 ± 0.0
HF		12545.9 ± 772.0	2964.8 ± 54.1	2.0 ± 0.0
CNTL	3'OMeEC-5-glucur	18463.3 ± 472.7	4203.6 ± 128.5	2.0 ± 0.0
HF		18345.4 ± 1540.4	4430.3 ± 71.0	2.0 ± 0.0

Data represented as mean ± SEM (n=3/group).

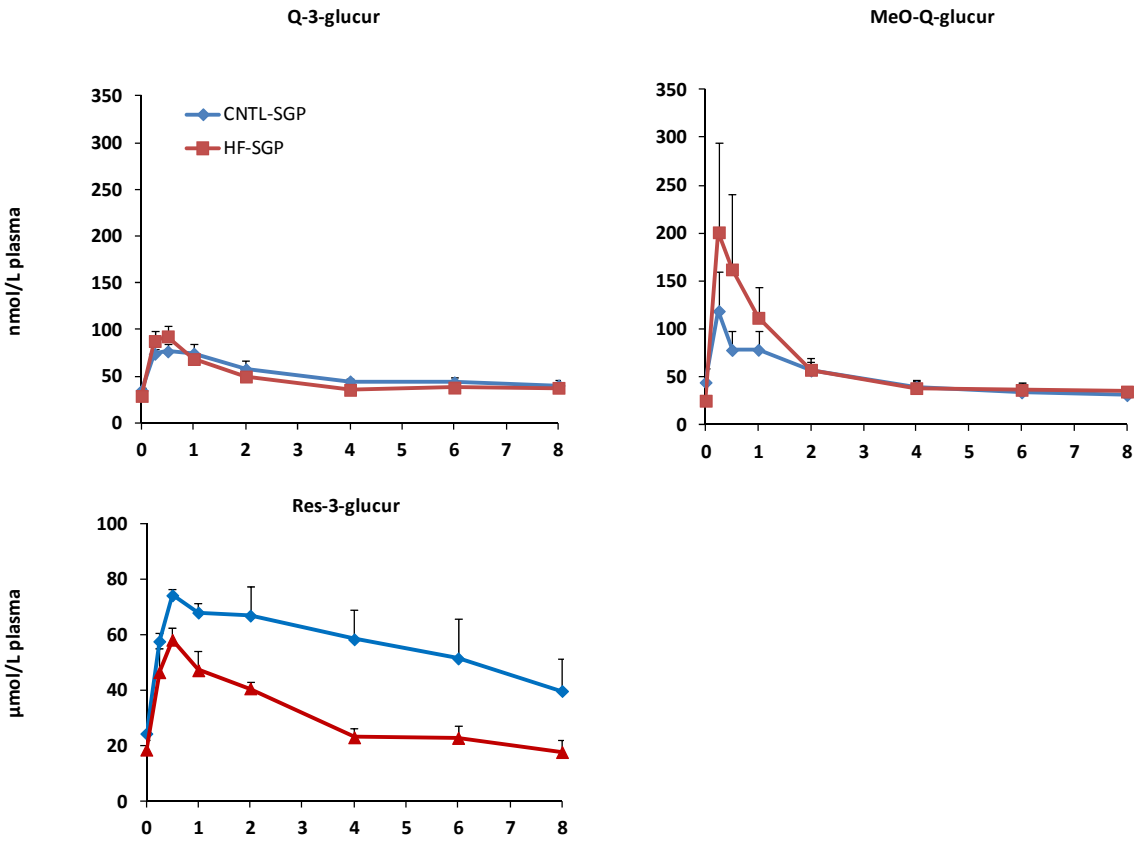


Figure 3.3. Plasma pharmacokinetic responses of quercetin and resveratrol metabolites. Data represented as mean ± SEM (n=3-4/group).

Table 3.4. Pharmacokinetic parameters of quercetin metabolites.

Diet	Compound	AUC (nmol/L-h)	C^{max} (nmol/L)	T^{max} (h)
CNTL	MeQ-Q-glucur	410.1 ± 43.9	78.8 ± 8.8	0.5 ± 0.2
HF		370.8 ± 14.8	95.7 ± 9.6	0.6 ± 0.2
CNTL	Q-3-glucur	385.4 ± 72.2	118.6 ± 41.5	0.3 ± 0.0
HF		467.4 ± 115.0	208.1 ± 90.3	0.3 ± 0.1

Data represented as mean ± SEM (n=4/group).

Table 3.5. Pharmacokinetic parameters of resveratrol metabolite.

Diet	Compound	AUC (µmol/L-h)	C_{max} (µmol/L)	T_{max} (h)
CNTL	Res-glucur	456.4 ± 76.0	78.5 ± 3.2	1.0 ± 0.5
HF		242.3 ± 32.0	58.2 ± 4.4*	0.5 ± 0.0

Data represented as mean ± SEM (n=4/group).

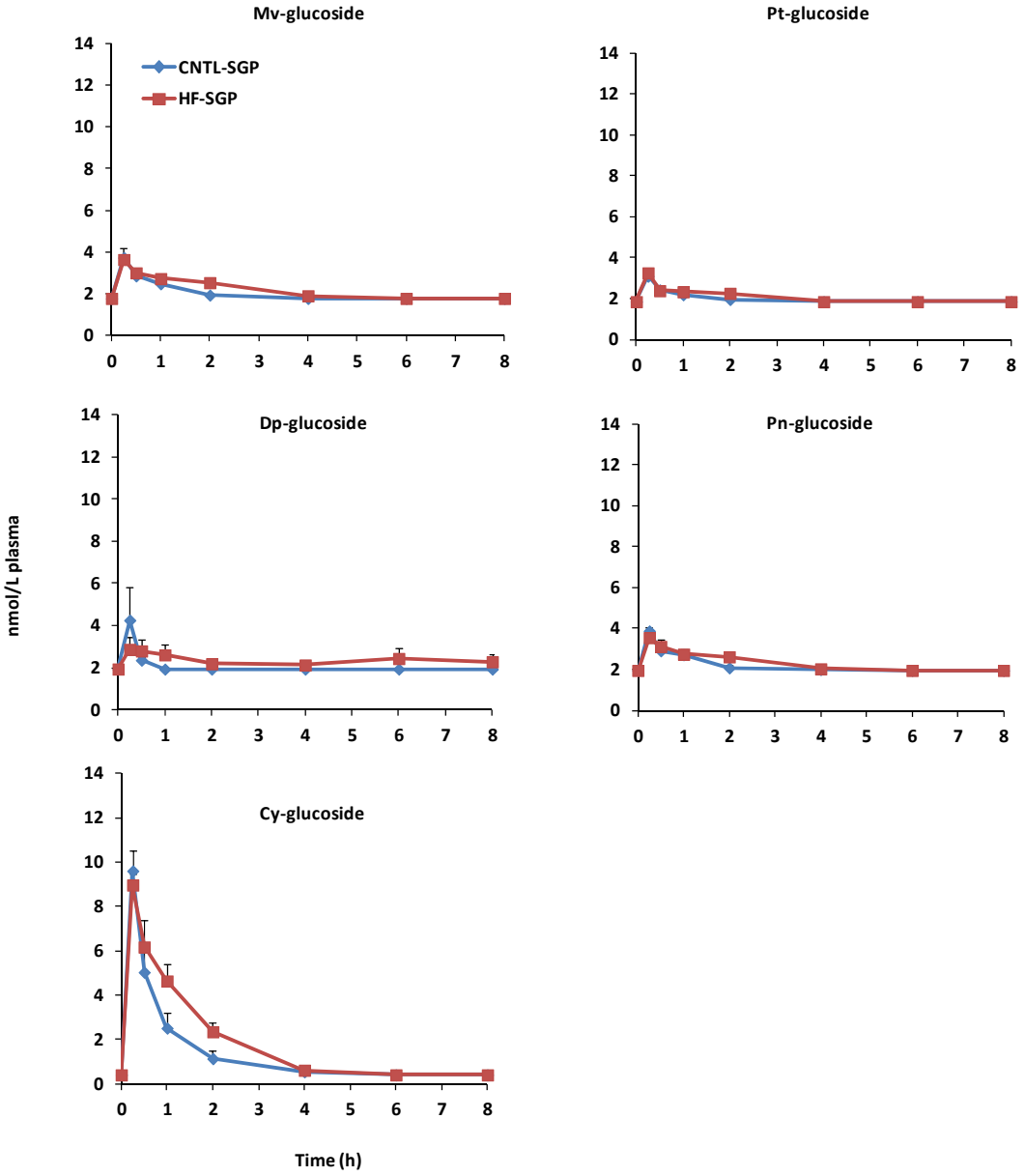


Figure 3.4. Plasma pharmacokinetic responses of major anthocyanins.
Data represented as mean ± SEM (n=4/group).

Table 3.6. Pharmacokinetic parameters of anthocyanins.

Diet	Compound	AUC (nmol/L-h)	C_{max} (nmol/L)	T_{max} (h)
CNTL	Mv-glucoside	15.9 ± 0.4	4.2 ± 0.4	0.3 ± 0.0
HF		17.3 ± 0.5	3.7 ± 0.1	0.3 ± 0.0
CNTL	Pt-glucoside	19.8 ± 4.1	3.1 ± 0.1	0.3 ± 0.0
HF		16.4 ± 0.3	3.3 ± 0.2	0.3 ± 0.0
CNTL	Dp-glucoside	16.8 ± 0.2	4.2 ± 0.1	0.3 0.0
HF		16.9 ± 0.3	4.0 ± 0.3	0.3 ± 0.0
CNTL	Pn-glucoside	17.4 ± 0.5	3.9 ± 0.2	0.3 ± 0.0
HF		18.3 ± 0.8	3.6 ± 0.3	0.3 ± 0.0
CNTL	Cy-glucoside	10.3 ± 1.5	9.6 ± 0.9	0.3 ± 0.0
HF		14.1 ± 1.0	9.0 ± 0.5	0.3 ± 0.0

Data represented as mean ± SEM (n=4/group).

SGP Polyphenol Metabolites in SD Brain Tissue.

Flavan-3-ols. Brain concentration of flavan-3-ol metabolites were determined to be at pico molar per gram of tissue range regardless the fat content in the background diet. Although rats fed on HF diet seemed to have slightly higher brain deposition levels on C-5-glucur, EC-5-glucur, 3'OMeC-5-glucur and 3'OMeEC-5-glucur, none of these effects reached the level of statistical significance compared to the CNTL diet group (Figure 3.5.). There were no flavan-3-ol metabolites detected in the water treated groups.

Quercetin. Brain concentration was detected at pM level for quercetin metabolites. The brain deposition levels for Q-3-glucur and MeO-Q-glucur were not significantly different between CNTL and HF diet group (Figure 3.6.).

Resveratrol. Brain deposition level was determined to be at nM level for Res-3-glucur which compares to its plasma level at micro molar per gram of tissue. There was no significant difference found between CNTL and HF diet groups (Figure 3.6.).

Anthocyanins. Brain concentrations were determined to be at pico molar per gram of tissue range for all major anthocyanins in SGP. Like other polyphenol in SGP, there was no significant difference detected in brain on anthocyanins between CNTL and HF diet groups (Figure 3.7.).

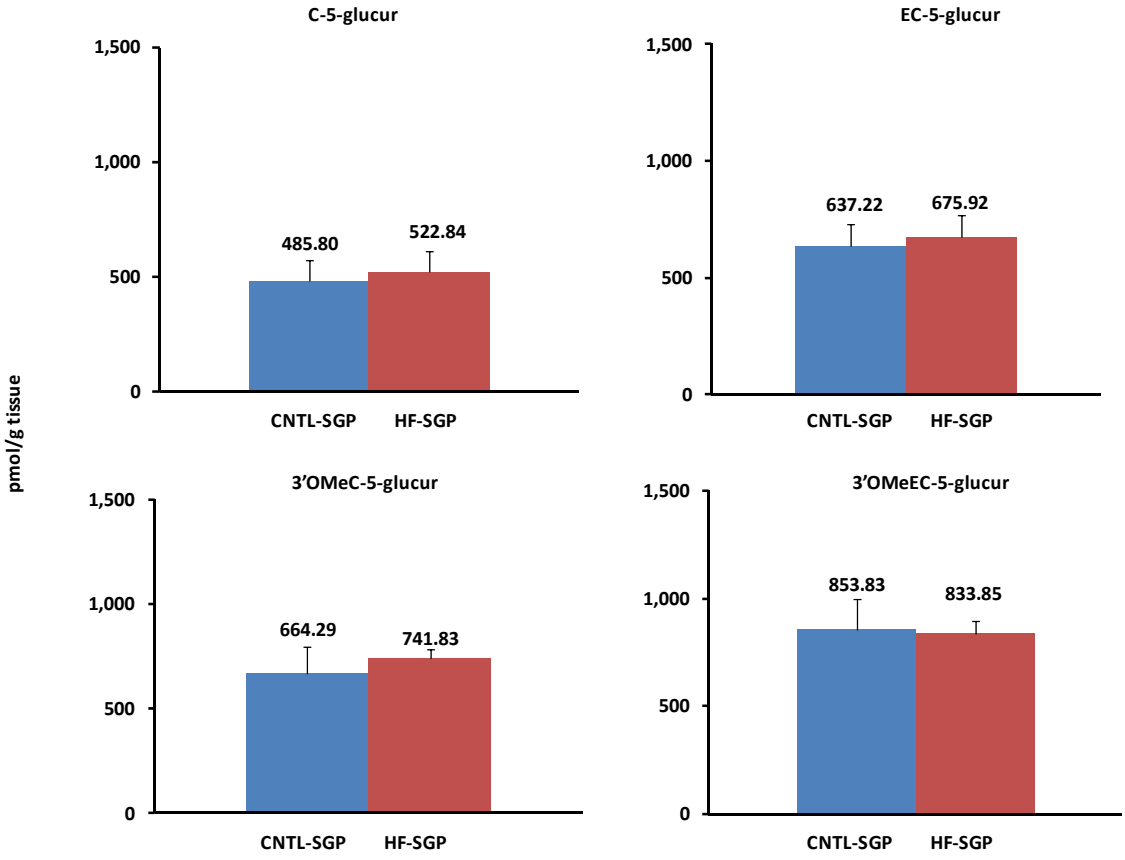


Figure 3.5. Brain responses of flavan-3-ols.
Data represented as mean ± SEM (n=3/group).

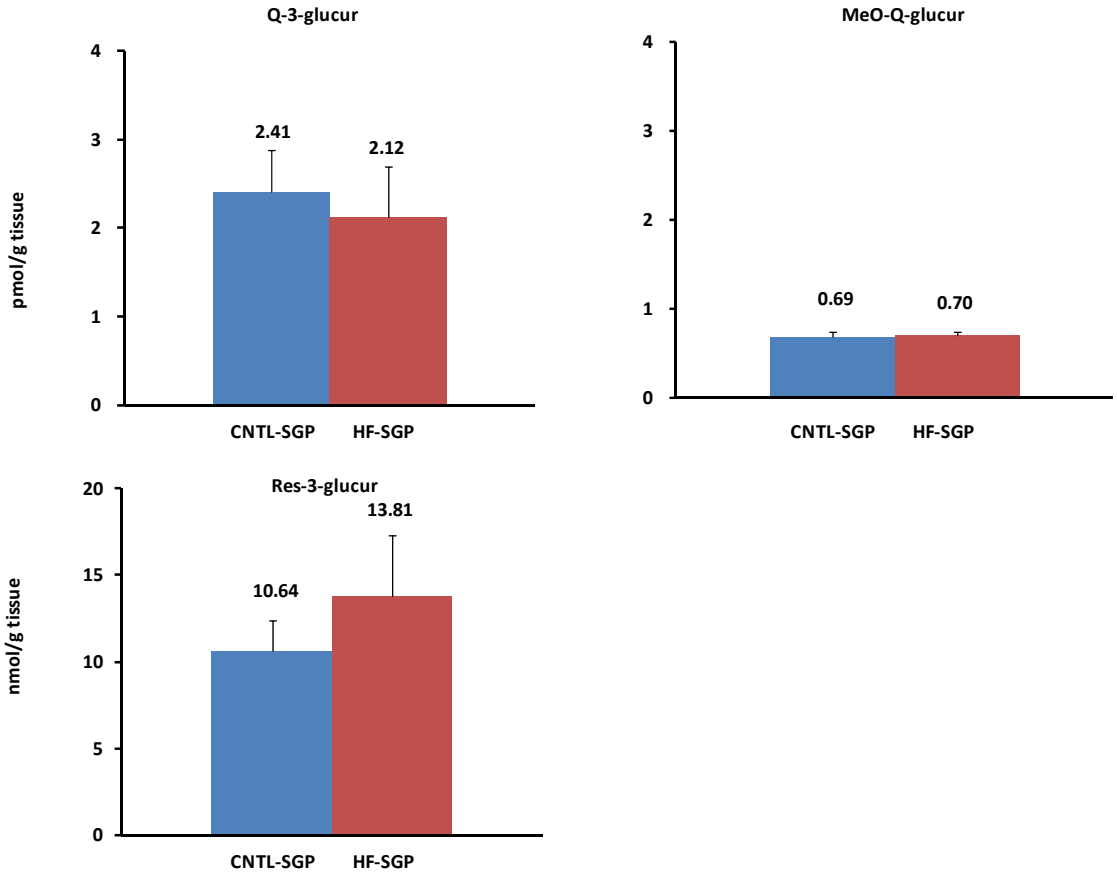


Figure 3.6. Brain responses of quercetin and reveratrol metabolites.
Data represented as mean ± SEM (n=3-4/group).

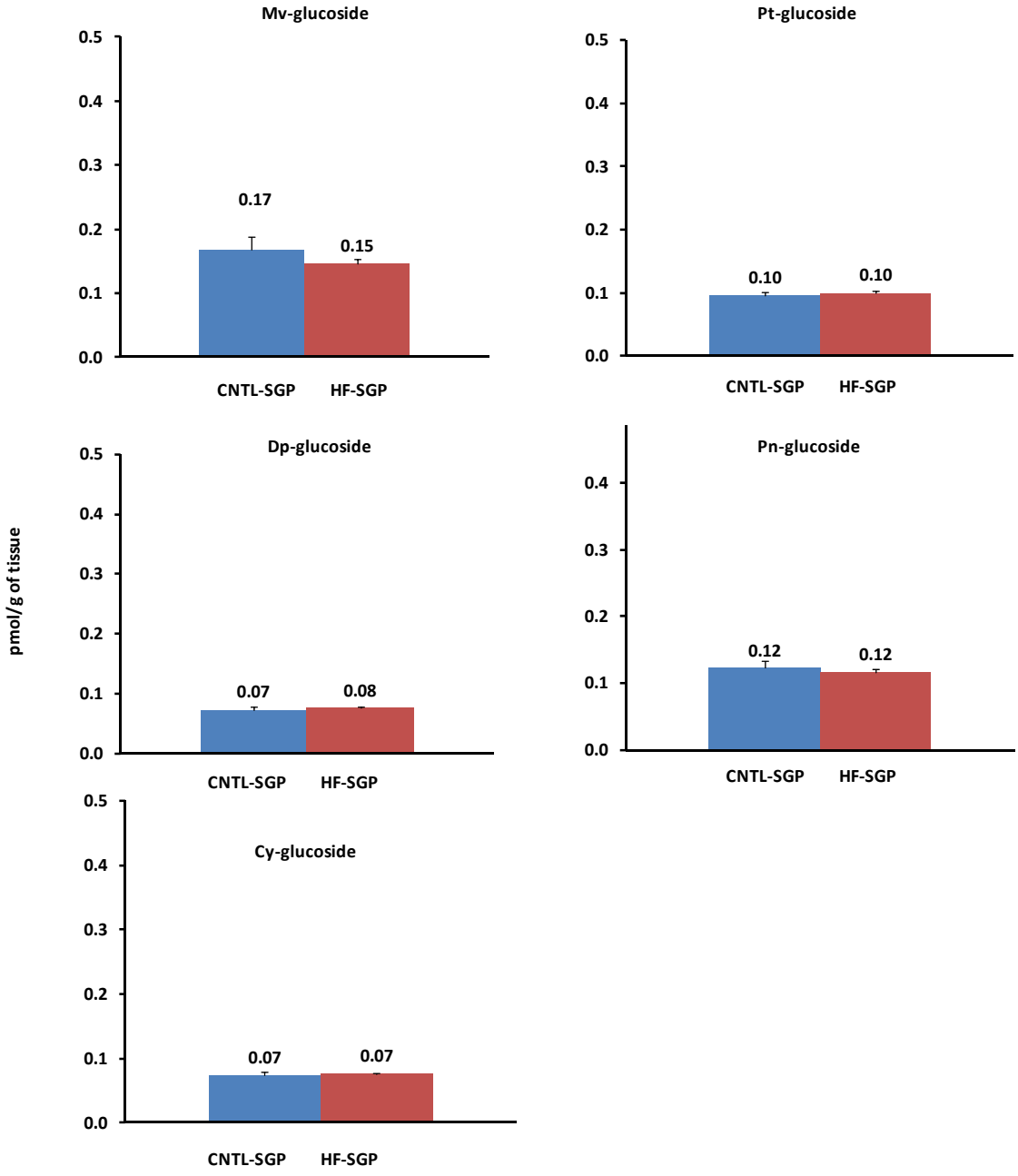


Figure 3.7. Brain responses of anthocyanins.
Data represented as mean ± SEM (n=4/group).

3.4 Discussion

Polyphenol metabolic profiles. We have previously demonstrated the bioavailability studies on a SD rat model gavaged individually with GSE, wine and resveratrol (Marambaud, Zhao et al. 2005; Ferruzzi, Lobo et al. 2009; Ho, Chen et al. 2009). The major polyphenols in these extracts are subjected to similar xenobiotic metabolism and are suspected to be the substrates for similar efflux proteins like p-glycoprotein, multidrug resistance-associated proteins and breast cancer resistance protein that limit their overall bioavailability (Monagas, Urpi-Sarda et al. 2010). It is reasonable to hypothesize that circulating and tissue profile of polyphenol metabolites might be modulated when treated simultaneously compared to individually. Our data suggested that similar metabolic profile was found when rats were gavaged with SGP versus individual polyphenol extract (Table 3.7.). Four flavan-3-ol metabolites including C-5-glucur, EC-5-glucur, 3'OMeC-glucur and 3'OMeEC-glucur were derived from C and EC which are the dominant components in GSE. Grape juice, which has similar composition to red wine, is enriched in quercetin-3-glucuronide and quercetin-3-glucoside which can be transformed to form Q-glucur and MeO-Q-glucur. Resveratrol is confirmed to be metabolized to Res-glucur regardless treated in combination with others or in individual form. These data confirm that when ingested in combination, polyphenol metabolic profile is not likely altered to a large extent. This supports the notion that combination treatment of polyphenols can be leveraged for future studies focused on AD prevention.

Table 3.7. Comparison of major polyphenol metabolites detected in Sprague-Dawley rats when treated individually or simultaneously.

SD rats	Individual polyphenol-rich product			SGP[4]
	GSE[1]	Concord grape juice[2]	Resveratrol[3]	
Flavan-3-ol	C-5-glucur EC-5-glucur 3'OMeC-5-glucur 3'OMeEC-5-glucur	N/A	N/A	C-5-glucur EC-5-glucur 3'OMeC-5-glucur 3'OMeEC-5-glucur
Flavonol	N/A	Q-3-glucur MeO-Q-glucur	N/A	Q-3-glucur MeO-Q-glucur
Anthocyanin	N/A	Mv-glucoside Pt-glucoside Dp-glucoside Pn-glucoside Cy-glucoside	N/A	Mv-glucoside Pt-glucoside Dp-glucoside Pn-glucoside Cy-glucoside
Resveratrol	N/A	N/A	Res-3-glucur	Res-3-glucur

1 (Wang, Ferruzzi et al. 2012)
2 in preparation
3 (Vingtdeux, Giliberto et al. 2010)

HF intervention and body weight change. In order to investigate how background dietary fat levels influences polyphenol absorption, SD rats were fed on 60% kcal% fat diet for 2 weeks prior to SGP gavage to induce condition the animals. However, our data did not show significant difference on major SGP polyphenol bioavailability between CNTL and HF background diet groups on brain levels as well as key PK parameters including C_{max} and $AUC_{0\text{-}8h}$. The exception to this was for EC-5-glucur and Res-3-glucur. It has been reported that SD rats respond to HF diet differently depending on their genetic background and also the training of their responsiveness to signaling and cues (Rada, Bocarsly et al. 2010). It has been suggested that within the SD rat species, they can be

grouping into obesity-prone and obesity-resistance (Levin and Keesey 1998; Dourmashkin, Chang et al. 2006). Another study showed that SD rats fed on 3 month of HF diet did not develop obesity or any other metabolic changes such as insulin resistance or glucose intolerance(Stark, Timar et al. 2000). In the present study, rats were not particularly trained to respond to cues associated with specific diet nor being characterized as obesity-prone or obesity-resistance and therefore, may be adaptive to HF diet to maintain body weight. The adaptation to HF may have manifested in lower food intake observed in the HF diet group compared to that in the CNTL diet group (Figure 3.1.) which suggested that SD rats might adapt to HF load in diet.

Overall, the unchanged body weight in SD rats well represented model of transient HF consumption in physiological healthy individuals. Furthermore, our data ruled out the potential confounding factor that the obese state might also influence polyphenol bioavailability (addressed in a different model in Chapter IV). There were reports showing that people lose weight when given HF nuts as snacks (Willette, Xu et al.). The possible mechanism was proposed by Mattes et al. (Mattes and Dreher ; Mattes, Kris-Etherton et al. 2008) that the fats were energy dense and highly satiable and therefore might decrease food intake. This was consistent with our data showing that HF diet groups showed a trend of decreased body weight change and food intake compared to the CNTL diet groups.

Difference in polyphenol bioavailability responded to HF intervention. In general, HF diet did not significantly influence polyphenol bioavailability, metabolism or brain distribution of compounds derived from SGP. With the exception of EC-5-glucur and Res-3-glucur, HF background diets seemed to result in improved plasma bioavailability of EC-5-glucur indicating by significantly higher $AUC_{0\text{-}8h}$ and C_{max} compared to CNTL (Table 3.2. and 3.3.). Our data suggested that fat content might help EC-5-glucur absorption. This is consistent with the study which showed that the higher fat content in cocoa liquor had better bioaccessibility compared to the lower fat cocoa powder due to a better micellarization of monomeric and dimeric flavan-3-ols (Ortega, Reguant et al. 2009). Interestingly, acute exposure to fat has been shown to improve bioavailability of other polyphenols including quercetin in humans (Guo, Mah et al. 2013). While similar effects may be possible in the current study, SGP dose treatments were not given to SD rats along with HF diet. Animals were in fact dosed after an overnight fast. Therefore, the mechanism behind this improvement in bioavailability for EC-glucur is not clear. More studies are needed to further explain such observation. On the other hand, C_{max} of Res-5- glucur was significantly higher in the CNTL diet group. It is suspected that excessive fat content might influence the expression of key metabolizing enzymes directly involved in polyphenol metabolism (Kim and Novak 2007). However, the mechanism should be further investigated.

Difference in brain penetration of polyphenol metabolites responded to HF intervention. Similar to plasma responses, effects of fat content in the background diet on brain concentrations of all four flavan-3-ol metabolites are limited. No significant differences were found on brain levels of quercetin, resveratrol metabolites and anthocyanins between HF and CNTL diets. Since the mixture of SGP contained different dosage of major polyphenol classes, we normalized the average C_{max}, AUC and brain concentration of key polyphenol metabolites by SGP dose in proportion to their concentration in the mixture (Table 3.8.). After the normalization process by SGP dose, the four major flavan-3-ol metabolites showed the highest brain levels at 200 nM/mg tissue compared to other metabolites. Interestingly, res-3-glucur showed a brain concentration at 13 nM/mg tissue even at a high dose of 56mg. These data suggested that flavan-3-ol metabolites are preferably selected to access brain tissues compared to other SGP polyphenols. This is indicative that flavan-3-ol metabolites are potent bioactive compounds with high brain targeting ability which can be recommended for further studies on AD prevention.

Table 3.8. Normalized C_{max}, AUC and brain levels by SGP dose.

SD rats on CNTL diet						Normalized by Dose		
Metabolite	Concentration in SGP (mg/g)	Dose (mg)	C_{max} (µmol/L)	AUC (µmol/L*h)	Brain (pmol/g)	C_{max} (µM/mg)	AUC (µM*h/mg)	Brain (nM/mg)
C-5-glucur	5.600	2.776	2.460	7.890	485.790	0.886	2.842	174.966
EC-5-glucur	6.490	3.218	2.790	9.120	637.220	0.867	2.834	198.033
3'OMeC-5-glucur	5.600	2.776	2.820	12.570	664.290	1.016	4.527	239.256
3'OMeEC-5-glucur	6.490	3.218	4.200	18.460	853.830	1.305	5.737	265.351
Q-3-glucur	0.090	0.045	0.119	0.385	2.410	2.658	8.636	54.009
MeO-Q-glucur	0.090	0.045	0.079	0.410	0.690	1.766	9.191	15.463
Mv-glucoside	0.014	0.007	0.004	0.016	0.170	0.602	2.294	24.491
Pt-glucoside	0.015	0.007	0.003	0.020	0.100	0.418	2.668	13.446
Dp-glucoside	0.030	0.015	0.004	0.017	0.070	0.282	1.129	4.706
Pn-glucoside	0.010	0.005	0.004	0.017	0.120	0.785	3.507	24.203
Cy-glucoside	0.023	0.011	0.010	0.010	0.070	0.843	0.901	6.139
Res-3-glucur	114.000	56.521	78.530	456.440	746.570	1.389	8.076	13.209

3.5 Conclusions

The goal of this study was to investigate the effect of transient HF consumption and the HF matrix on grape polyphenol bioavailability and brain deposition level. While bioavailability of flavonoids can be affected by macronutrient content (Ferruzzi 2010; Bandyopadhyay, Ghosh et al. 2012), few studies have investigated the direct effect of dietary fat content on polyphenol bioavailability. This study was the first to our knowledge that tested the bioavailability on previously proven effective grape polyphenols, GSE, grape juice and resveratrol, on SD rats fed on CNTL or HF diet for 3 weeks. We have found that different background diets with high or low fat contents showed limited effect on plasma bioavailability of individual flavan-3-ol metabolites with the exception of EC-5-glucur and Res-3-glucur. Further brain levels of individual metabolites of SGP did not differ regardless of the background diet. In conclusion, our data suggested that the transient high fat ingestion for up to 3 weeks on physiologically

healthy rats did not significantly influence the overall plasma bioavailability and brain levels of polyphenols from an SGP preparation.

CHAPTER 4. INFLUENCE OF DIABETES ON PLASMA PHARMACOKINETICS AND BRAIN BIOAVAILABILITY OF GRAPE POLYPHENOLS IN THE ZUCKER DIABETIC FATTY RAT MODEL

Abstract

Grape polyphenols are believed to be biologically active agents capable of modulating Alzheimer's disease (AD) risk factors. Diabetes mellitus is a known risk factor for development of AD. To investigate the effect of the diabetic condition on bioavailability and brain distribution of grape-derived flavan-3-ols, flavonols, and anthocyanins, Zucker diabetic fatty (ZDF) rats and their lean controls (LN) were dosed orally with a combination of grape polyphenols termed Standardized Grape Polyphenol (SGP) which consisted of grape seed extract (GSE), Concord grape juice and resveratrol (Res). Each component had previously been found effective in prevention of AD in a mouse model (Wang, Ho et al. 2008; Vingtdeux, Giliberto et al. 2010). Eight hour plasma pharmacokinetics was conducted after a 10-day oral gavage treatment of SGP or water. LC-MS/MS analysis confirmed metabolites of catechin (C) and epicatechin (EC) as C/EC-5-glucuronide (C/EC-5-glucur) and 3'-methyl-C/EC-5-glucuronide (3'-OMeC/EC-5-glucur). The main metabolites of quercetin (Q) present in plasma and brains were determined to be Q-3-glucuronide (Q-3-glucur) and methyl Q-glucuronide (MeO-Q-glucur). Res-3-

glucuronide (Res-3-glucur) was the main metabolite identified derived from resveratrol in plasma and brain extracts.

ZDF rats exhibited significantly diminished C_{max} for C/EC-5-glucur (p=0.01 and p=0.008), 3'-OMeC/EC-5-glucur (p<0.0001), Q-3-glucur (p=0.01), MeO-Q-glucur (p=0.006) and Res-3-glucur (p=0.003). Bioavailability defined as plasma area under the curve (AUC) was found to be significantly lower in ZDF rats for 3'-OMeC-5-glucur (25.09±13.47 vs. 108.09±6.65 µmol/L*h, p<0.0001), 3'-OMeEC-5-glucur (22.95±12.40 vs. 110.26±5.94 µmol/L*h, p<0.0001), Res-3-glucur (180.78±20.54 vs. 328.85±40.53 µmol/L*h, p=0.006), Q-3-glucur (28.63±2.73 vs. 50.72±7.26 nmol/L*h, p=0.02) and MeO-Q-glucur (71.86±3.21 vs. 123.03±19.85 nmol/L*h, p=0.03) than the LN rats. Additionally, ZDF rats showed significantly lower levels in all stilbene, flavanol and flavan-3-ol metabolites found in brain tissues. There was no significant differences in anthocyanins in plasma between ZDF and LN, and no anthocyanins were detectable in brain extracts. ZDF rats showed significantly higher urinary excretion for all polyphenols from SGP. These data suggested that the diabetes negatively impact absorption and brain distribution of polyphenol metabolites *in vivo,* potentially due, in part, to higher urinary clearance of absorbed metabolites. This study suggests that dosages required to reach therapeutic efficacy may need to be adjusted based on the presence and/or severity of diabetes.

4.1 Introduction

AD is an age-related neurodegenerative disease projected to affect more than 5 million Americans by the year 2050 (Hebert, Scherr et al. 2003) with an estimated cost of $148 billion (Alzheimer's 2009). Despite its severity and economic burden, there is currently no cure. Diabetes mellitus is a condition defined by hyperglycemia which leads to high risks of microvascular damage including retinopathy, nephropathy and neuropathy (WHO 2006). Diabetes affected 25.8 million people in the United States in 2010 which accounted for 8.3% of the U.S. population (CDC 2011). Like AD, diabetes poses a huge economic burden on the society with $174 billion dollars medical costs estimated in 2007 (CDC 2011).

Sedentary life style and high fat consumption are believed to contribute to the development of metabolic syndrome which may eventually progress to diabetes. In a 27 year longitudinal population-based study, obesity and body mass index has been strongly associated with onset of dementia and AD (Whitmer, Gunderson et al. 2005). The same group also highlighted that body mass index was a risk factor for AD (Whitmer 2007; Whitmer, Gunderson et al. 2007). Disturbances in insulin signaling in diabetes have been suggested to be a contributing factor for AD development and progression (Li and Holscher 2007). The Rotterdam study suggested that insulin resistance increased the chances of progression to AD at a 3 year follow up by 40% (Schrijvers, Witteman et al.). In a recently published four year longitudinal study, researchers found that insulin resistance in a late-middle aged cohort is positively associated with brain atrophy in the

regions that are affected by early AD. Their results suggested that higher insulin resistance predicted medial temporal lobe atrophy associated with cognitive deficits (Willette, Xu et al.). Considering the link between the diabetic state and neurodegenerative process, diabetic and metabolic syndrome patients are likely targets for AD preventive treatments.

Individual grape derived products including purple grape juice, red wine, grape skin and seed extracts have been shown to have biological activities consistent with the prevention or amelioration of diabetes and AD in rodent models (Marambaud, Zhao et al. 2005; Wang, Ho et al. 2006; Wang, Ho et al. 2008; Chis, Ungureanu et al. 2009; Wang, Thomas et al. 2009; Drel and Sybirna 2012; Huang, Tsai et al. 2012). These polyphenol rich products provide distinct polyphenol profiles including flavan-3-ols, flavonols, and stibenoids such as resveratrol. While present in the extract, conjugated metabolites of these parent polyphenol forms have been identified in circulation and in brain tissue (Ferruzzi, Lobo et al. 2009; Vingtdeux, Giliberto et al. 2010; Ho, Ferruzzi et al. 2012; Wang, Ferruzzi et al. 2012). These metabolites including both methylated and glucuronidated conjugates have demonstrated the ability to target distinct molecular mechanisms related to AD (Vingtdeux, Giliberto et al. 2010; Ho, Ferruzzi et al. 2012; Wang, Ferruzzi et al. 2012). For example, 3'-O-methyl-epicatechin-5-glucuronide, a metabolite derived from flavan-3-ol in grape seed extract have been shown to restore synaptic plasticity in the brain of AD mice (Wang, Ferruzzi et al. 2012). Resveratrol has been shown to activated AMPK pathway which in term rediced cerebral Aβ levels in AD

mice (Vingtdeux, Giliberto et al. 2010). These observations stimulate interests in devising combination strategies favoring simultaneous delivery of all bioactive brain targeting polyphenol metabolites to brain tissues. Interestingly, impaired insulin signaling, a major defect of type II diabetes is believed to alter expression of key metabolizing enzymes responsible for generation of bioactive polyphenol metabolite forms including UDP-glucuronosyltransferase and sulfotransferases (Kim and Novak 2007). Additionally, the diabetic condition may alter gastrointestinal mobility and endogenous hormone secretion in diabetes that can, in turn, impact polyphenol absorption and metabolism rate (Samsom, Vermeijden et al. 2003). Diabetes has been shown to alter the function of enteric nervous system and the secretion and sensitivity of many key hormones (insulin, glucagon and growth hormone) leading to modifications in xenobiotic metabolizing system (Chandrasekharan and Srinivasan 2007).

Another complication caused by diabetes is osmotic dieresis and electrolyte imbalance resulting from hyperglycemia. Osmotic dieresis results in urinary loss of water and electrolytes and if prolonged can progress to kidney failure (Oh, Joo et al. 2007). Polyphenols undergoing xenobiotic mechanism are rapidly excreted through urinary routes. Considering the excessive urinary loss in diabetic animals, the potential impact to circulating polyphenol metabolites and ultimate delivery to brain tissues may be significant. Therefore, while the diabetic condition may be a risk factor for AD onset and progression, it may also require alteration of therapeutic or preventive strategies due to altered polyphenol bioavailability.

In order to better understand the impact of the diabetic condition on polyphenol bioavailability and metabolism, a study examining the pharmacokinetic behavior and brain deposition of grape derived polyphenol metabolites from SGP mixture of Concord grape juice, GSE and resveratrol was assessed in both lean and obese ZDF rats.

4.2 Materials and Method

Chemicals and Materials.

(+)-C, (-)-EC and Q-3-glucur authentic standards were purchased from Sigma-Aldrich (St. Louis, MO). All extraction and liquid chromatography solvents were HPLC certified and were obtained from J.T. Baker (Phillipsburg, NJ). GSE MegaNatural® AZ powder was provided by Polyphenolics (Madera, CA). 100% Concord grape juice (Welch's) was loaded onto C18 Solid Phase extraction cartridges (1 c.c.) and washed with ddH_2O (0.01% HCl) to remove sugar. Polyphenols were then eluted with MeOH (0.01% HCl). MeOH was then removed under vacuum and the resulting concentrate was kept frozen until use. Resveratrol, Res-3-glucur, malvidin (Mv)-3-glucoside chloride and cyanidin (Cy)-3-glucoside chloride were purchased from ChromaDex (Irvine, CA).

SGP Material Analysis.

Major polyphenol contents in SGP were determined by Rutgers University with the method described in Chapter 3.

Animals.

All animal studies were approved by the Purdue University Animal Care and Use Committee. Twelve 10 week old male ZDF rats and twelve 10 week old male LN rats were obtained from Charles Rivers Labs (Wilmington, MA). Rats were placed on AIN-93M polyphenol free diet using corn oil replacing soybean oil (Dyets, Bethleham, PA) with water ad libitum until 13 weeks of age. Diabetes was at an advanced stage in the ZDF rats at 13 weeks of age. Blood glucose after an 8 h fast was measured by AlphaTRAK® glucose meter from Abbott Laboratories (32004-02, Abbott Park, IL) the day before SGP treatment and on the pharmacokinetics day. Blood glucose level higher than 250 mg/dL is considered to be hyperglycemia. Body weight and food intake were monitored every other day throughout the study.

SGP Pharmacokinetics.

The animal study design was modified from Ferruzzi et al. (Ferruzzi, Lobo et al. 2009) with minor changes. In brief, 13 weeks old rats were randomly assigned to ZDF - control (CRTL) (n=4), ZDF - SGP (n=8), LN - CRTL (n=4) and LN - SGP (n=8). Rats were placed in metabolic cages for urine collection and were dosed daily for 10 days with SGP at a dose of 150 mg/kg BW of GSE, 100 mg/kg BW of Concord grape juice and 297 mg/kg BW of trans-resveratrol (based on total polyphenolic content shown in Table 4.1.). Rats in the CNTL groups received water. After SGP treatment on the eighth day, rats were anesthetized with 3-5% of isoflurane and a polyethylene catheter was surgically

implanted into the jugular vein for blood sampling. Rats were injected with Buprenex (0.05 mg/kg) before regaining consciousness to alleviate pain. Catheters were kept patent by flushing with ~0.3 mL of heparinized saline (100 units/mL) every 12 h. Rats were allowed to Rest for 24 h to recover after surgery.

Pharmacokinetics was initiated on the tenth day after 8h of fast and food was returned 2 h after SGP dosage. DI water was added to SGP to a final volume of 1.0 mL. Residual doses were rinsed with 0.5 mL DI water and administered to rats as the second gavage. Rats were gavaged twice a day, 8 h apart with half the daily dosage. 12h urine samples were collected daily via metabolic cages right after daily SGP treatment.

Table 4.1. Total polyphenolic content of Concord grape juice, GSE and resveratrol.

Total Polyphenolic Content	**Grape Juice**	**GSE**	**Resveratrol**
wt/wt %	**8.8**	**94**	**98**

Pharmacokinetic studies were conducted on the 10th day of SGP treatment by collecting 400 µL of heparinized blood at 0, 0.25, 0.5, 1, 2, 4, 6, 8 h post gavage from the jugular catheter. Heperinized blood was centrifuged at 6500 rpm for 10 min at 4 ℃ Resulting in 200 µL of plasma. Plasma was acidified with acidified saline (1% ascorbic acid wt/v) in 4:1 ratio, purged with nitrogen and stored at -80 ℃ until analysis. The day after pharmacokinetics, another dose was administered and rats were sacrificed 1 h post dose. Rats were then perfused with ice-cold saline to remove residual blood from

tissues. Brain tissues were harvested following perfusion and snap frozen in liquid nitrogen.

Polyphenol Metabolites Extraction in Plasma, Urine and Brain Tissues.

C/EC, Q, Res metabolites and anthocyanins were extracted from plasma, urine and brain homogenates by solid phase extraction (SPE) using 1 mL Waters Oasis HLB cartridges (Milford, MA). The protocol was previously described by Ho et al. (Ho, Ferruzzi et al. 2012). In brief, acidified plasma, urine and methanolic brain extracts were loaded onto preconditioned SPE cartridges. The cartridges were washed with 1 mL of 1.5M formic acid (v/v) followed by 1 mL of 5% aqueous methanol (v/v) for C/EC, Q, Res metabolites, 2 mL of 2% formic acid (v/v) for anthocyanins. C/EC, Q, Res metabolites were eluted with 2 mL of 0.1% formic acid/methanol (v/v) and anthocyanins were with 2% formic acid/methanol (v/v). Eluents were dried under vacuum at 37 ℃. Dried extracts were reconstituted with LC mobile phases for immediate analysis. Plasma from two rats was combined as one sample to undergo anthocyanin SPE due to their low concentration in these tissues and fluids.

Polyphenol Analysis of Plasma, urine and Brain Tissues by LC-MS or LC-MS/MS.

Analyses of C/EC, Q, Res metabolites was performed on an Agilent 6400 Triple Quad LC/MS equipped with an electron spray ionization (ESI) source under multiple reaction monitoring modes (MRM). The method was previously described by Ho et al. (Ho,

Ferruzzi et al. 2012). Briefly, a Waters XTerra RP-C18 column (2.1 mm x 100 mm, 3.5 µm particle size) was employed for all analyses. For C/EC, Q and Res, binary mobile phases were A: 0.1% aqueous formic acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v). The column was heated to 30℃ and the system flow rate was 0.3 mL/min. The binary gradient to elute all polyphenol metabolites was: 10% B at 0 min, 40% B at 10 min, 95% B at 11 min and back to 10% B at 12 min to 18 min. Fragmentor voltage was set at 135V and collision energy was 17eV for all compounds. ESI source conditions were as follows: gas temperature was 350℃, drying gas flow was 11 l/min, nebulizer was 30 psi, sheath gas temp was 350℃, sheath gas flow was 11 L/min, capillary voltage was 3500V and nozzle voltage was 1000V. For anthocyanins, the binary mobile phases were A: 2% aqueous formic acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v) and the column was heated up to 35℃. The gradient used was: 5% B at 0 min, 10% B at 10 min, 25% B at 30 min, 5% B at 31 min and continue on 5% B to 35 min. ESI source condition setting was the same as described above.

Flavan-3-ols and resveratrol metabolites in urine samples were analyzed by Agilent 1100 LC-time of flight equipped with ESI source. LC conditions were the same as described above as used in LC-MS/MS. MS conditions: gas temperature was 350℃, drying gas flow was 11 l/min, nebulizer was 30 psi, capillary voltage was 3500V, skimmer was 60V, fragmentor was 135V and the mass range was 100-1000 m/z. Quercetin metabolites and anthocyanins were analyzed by triple quadrupole as described above.

Data Analysis.

Quantification of C/EC, RES and Q metabolites was by multi-level response curves generated from authentic standards of C, EC, Res-3-glucur and Q-3-glucur, respectively. Quantification of Cy-3-glucoside was calculated from a calibration curve constructed with authentic standard while other anthocyanins were based on Mv-3-glucoside. All data are presented as mean ± standard error of the mean (SEM). Pharmacokinetic parameters including: 1) area under the plasma concentration versus time ($AUC_{0\text{-}8h}$) were calculated using the linear trapezoidal rule, 2) the maximum plasma concentrations (C_{max}) and, 3) the time at which the maximum concentration (T_{max}) were determined directly from the pharmacokinetic curves of plasma concentration versus time. Statistical analyses were performed using SAS 9.3 statistical analysis program (Cary, NC). Group differences of average body weight gain, average food intake and the difference of fasting blood glucose before and after treatment were determined by one-way ANOVA with Tukey's post-hoc test. Differences between ZDF and LN on pharmacokinetic parameters, urine concentration and brain concentrations were analyzed by Student's t test. The significance was accepted at the level of $\alpha<0.05$.

4.3 Results

Body Weight, Food Intake and Fasting Blood Glucose

Body weight and food intake of all groups were measured every other day (Figure 4.1.). Body weight gain was defined by the body weight difference between the first day

of treatment and the day of jugular surgery. One of the ZDF-CNTL rats lost ~51g of body weight during the course of water treatment resulting in negative average body weight gain. This might be due to the severity of diabetes complication progressed as the study went on. In general, treatment of SGP did not significantly alter body weight gain in either ZDF or LN rats. SGP also did not have significant effects on average food intake on LN or ZDF rats. Fasting blood glucose was measured before and after 10 days of SGP treatment. LN-SGP rats had average fasting blood glucose of 107.38 ± 2.07 mg/dL before SGP treatment and 111.57 ± 2.82 mg/dL after 10 days of SGP treatment. ZDF-SGP rats, on the other hand, had average fasting glucose level of 234.38 ± 16.22 mg/dL prior to SGP and 300.17 ± 40.89 mg/dL after 10 days of SGP treatment. LN-CNTL rats had fasting blood glucose of 114.25 ± 1.65 before and 116.75 ± 1.65 mg/dL after 10 days of water treatment. ZDF-CNTL rats had fasting blood glucose at 262.50 ± 30.64 before and 346.25 ± 19.98 mg/dL after water treatment. The difference of fasting blood glucose before and after treatment was not significantly different in either diabetic or lean groups suggesting that 10-day of SGP treatment did not significantly influence fasting blood glucose level.

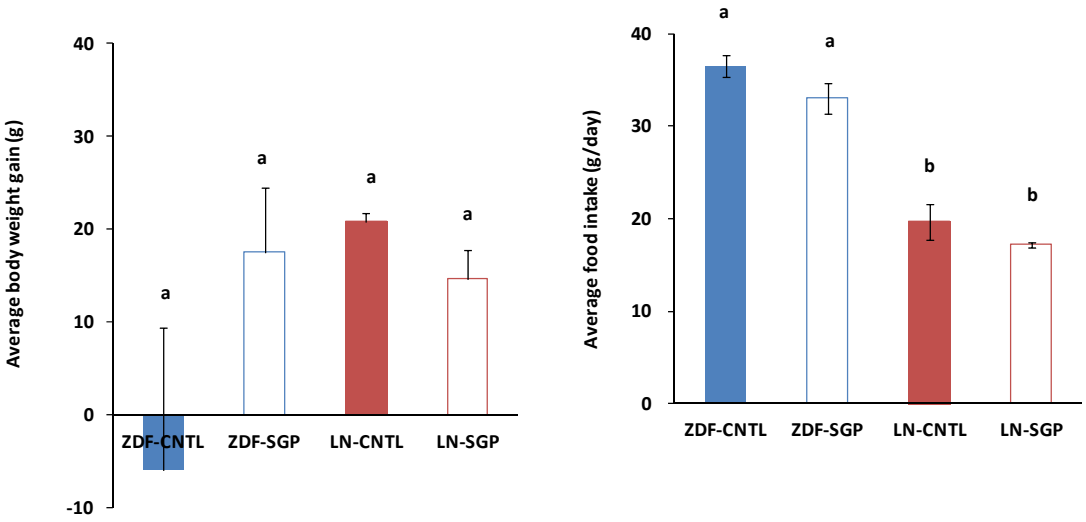


Figure 4.1. Average body weight gain and average food intake of rats.
Data was represented as mean ± SEM (n=4/CNTL group and n=8/SGP group).
Different letter indicates significance among groups.

Characterization of Major Polyphenols in Plasma and Brain Tissues

Four major flavan-3-ol metabolites, C-5-glucur, EC-5-glucur, 3'-OMeC-5-glucur, and 3'-OMeEC-5-glucur were detected in plasma and brain tissues from rats treated with SGP (Figure 4.2.). Res-3-glucur was the main metabolite from resveratrol found in plasma and brain tissues (Figure 4.2.). Chemical structures of major SGP polyphenols and metabolites are shown in Figure 4.3. Major Q metabolites were Q-3-glucur and MeO-Q-glucur (structures shown in Figure 4.3.) and representative chromatograms are shown in Figure 4.4. This finding was also consistent with our previous data on SD rats treated solely with Concord grape juice (Chen, Lobo et al. 2011). A comparison of major SGP polyphenol metabolites are listed in Table 4.2.

There was a major peak shown in the brain extract tentatively assigned as a Q-glucur based on MRM Response (Figure 4.4.) and in-line spectra (data not shown). However,

assignment of the glucuronidation site was not possible at this time as isolation and NMR analysis is required for complete assignment. All major polyphenol metabolites including C-5-glucur, EC-5-glucur, 3'-OMeC-5-glucur, 3'-OMeEC-5-glucur, Res-3-glucur, Q-3-glucur and MeO-Q-glucur were found in the extracts of plasma and brains for both ZDF rats and their LN counterparts. No metabolites were detected in plasma and brain extracts from control rats.

Major anthocyanins detected in plasma were malvidin (Mv), petunidin (Pt), peonidin (Pn), cyanidin (Cy) and delphinidin (Dp) glucosides with structures depicted in Figure 4.3. MRM chromatograms of anthocyanins from plasma were shown in Figure 4.5. There were no detectable levels of anthocyanidin metabolic derivatives in the brain tissues of SGP or control animals.

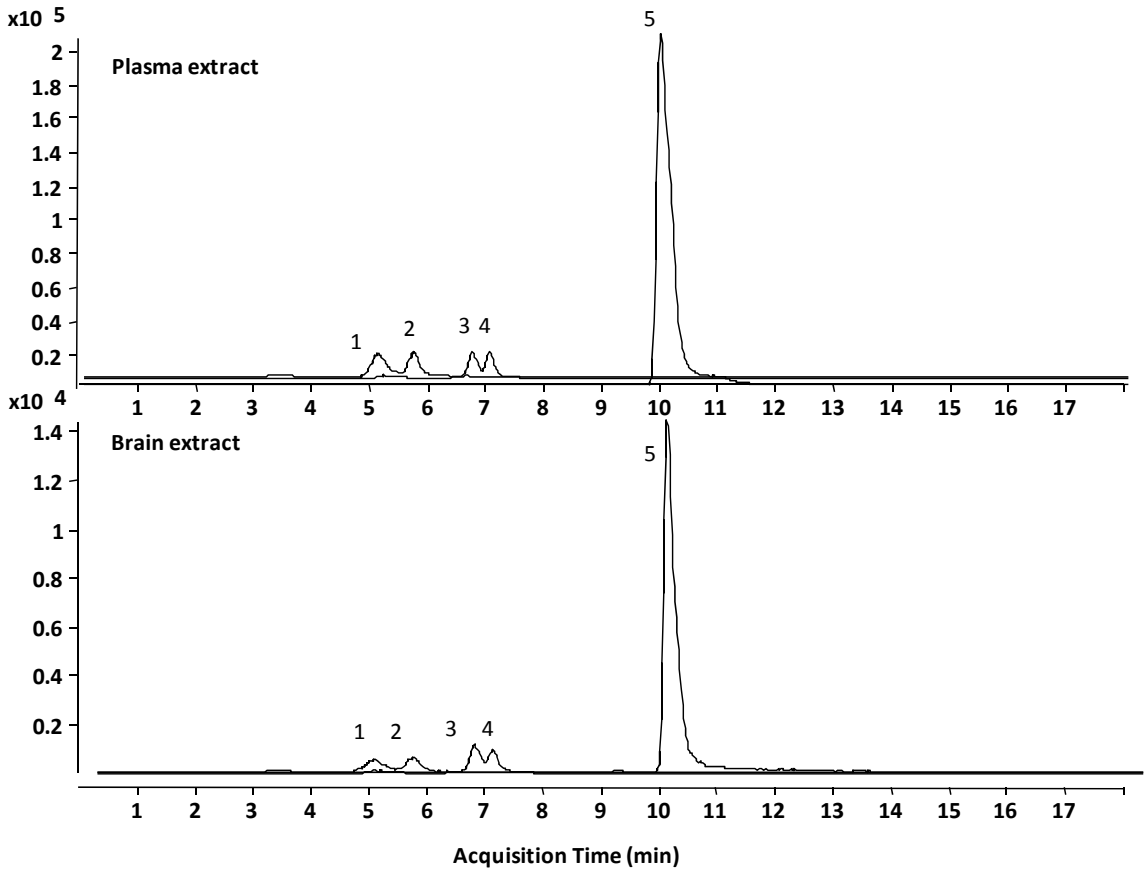
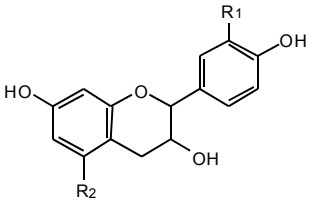
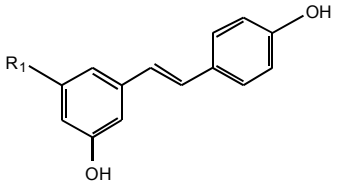


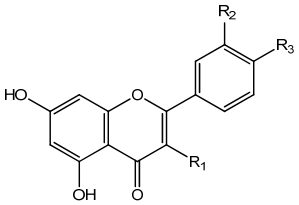
Figure 4.2. Representative MRM chromatograms of major flavan-3-ol and resveratrol metabolites from plasma and brain extracts.
MRM chromatogram is shown for C/EC-5-glucur (465.1 -> 289.1 m/z), 3'-MeO-C/EC-5-glucur (479.1 -> 303.1 m/z) and Res-3-glucur (403.1 -> 227.1 m/z) under negative polarity. Peak identifications are: 1. C-5-glucur, 2. EC-5-glucur, 3. 3'-OMeC-5-glucur, 4. 3'-OMeEC-5-glucur and 5. Res-3-glucur.



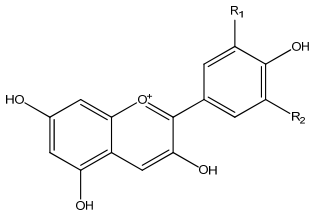
Flavan-3-ols	R_1	R_2
C/EC	OH	OH
C/EC-5-glucur	OH	O-glucuronicacid
3'-OMe-EC/C-5-glucur	OCH_3	O-glucuronicacid



Res Metabolite	R_1
Res	OH
Res-3-glucur	O-glucuronicacid



Q Metabolite	R_1	R_2	R_3
Q	OH	OH	OH
Q-3-glucur	O-glucuronic acid	OH	OH



Anthocyanin	R_1	R_2
Cy-gluc	OH	H
Mv-gluc	OCH_3	OCH_3
Dp-gluc	OH	OH
Pn-gluc	OCH_3	OH
Pt-gluc	OCH_3	OH

Figure 4.3. Chemical structures of SGP metabolite detected in plasma and brain extracts.

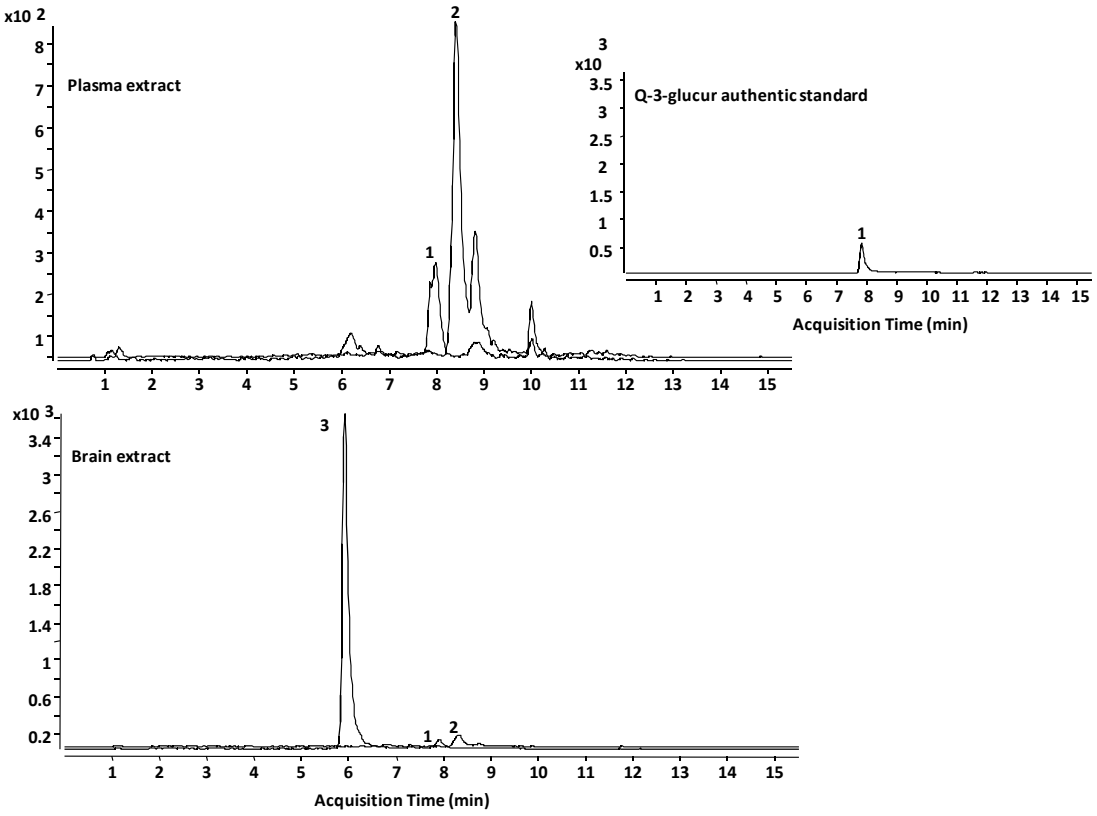


Figure 4.4. Representative MRM chromatograms of major Q metabolites from plasma and brain extracts.
MRM trace is shown for Q-glucur (479.1 -> 303.1 m/z) and MeO-Q-glucur (493.1 -> 317.1 m/z). Peak identifications were: 1. Q-3-glucur, 2. MeO-Q-glucur and 3. Tentatively identified as Q-glucur with glucuronidation in an unknown position.

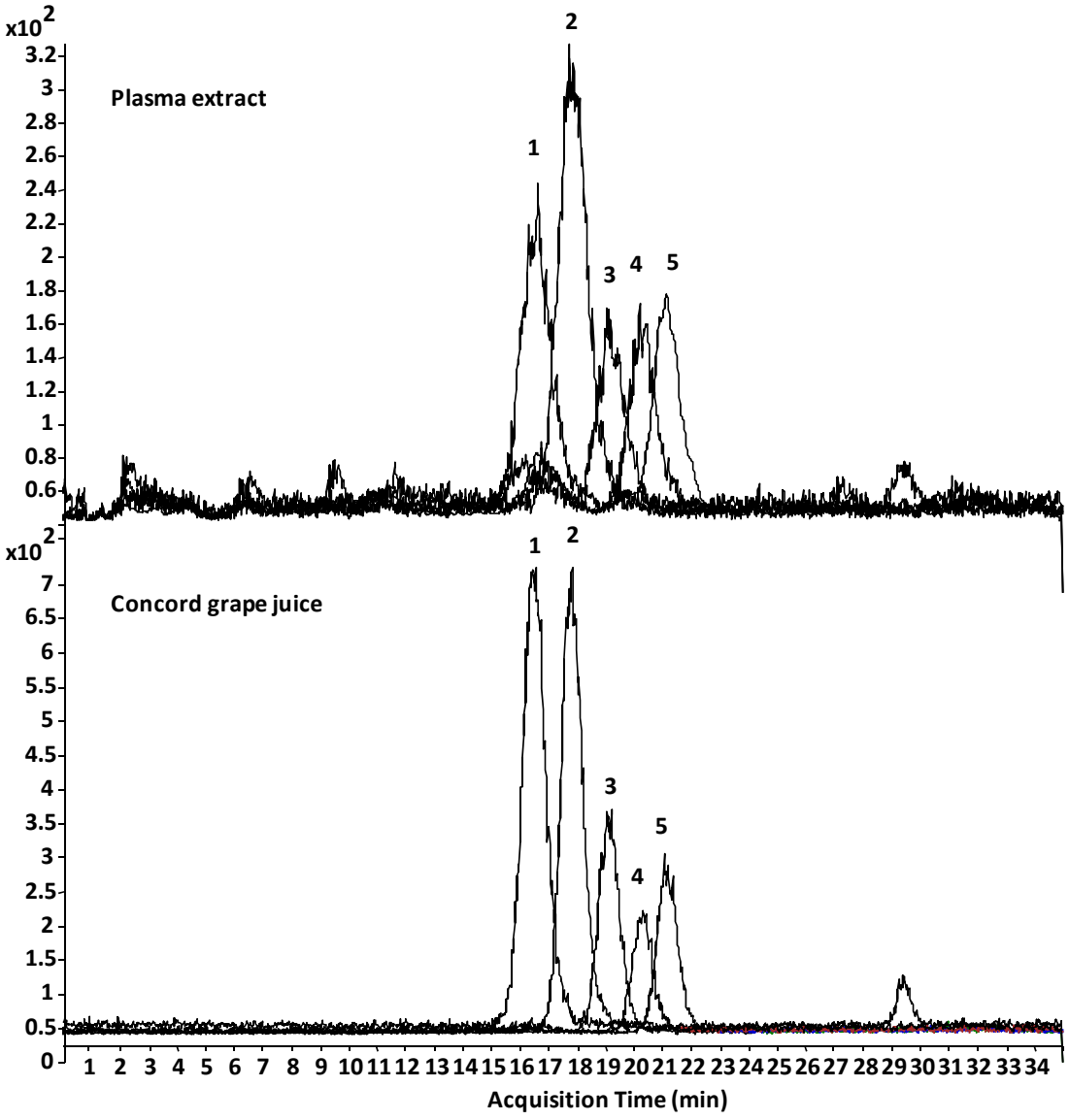


Figure 4.5. Representative MRM chromatograms of anthocyanin derivatives from plasma extracts and Concord grape juice.
MRM transitions were: 493.1 -> 331.1 for Mv-3-glucoside, 479.1 ->317.1 for Pt-glucoside, 465.1 -> 303.1 Dp-glucoside, 463.1 -> 301.1 for Pn-glucoside and 449.1 -> 287.1 for Cy-3-glucoside. Peak identifications were: 1. Mv-3-glucoside, 2. Pt-glucoside, 3. Dp-glucoside, 4. Pn-glucoside and 5. Cy-3-glucoside.

Table 4.2. Comparison of major polyphenol metabolites detected in SD rats and ZDF rats treated with SGP.

SGP	SD rats	ZDF rats
Flavan-3-ol	C-5-glucur	C-5-glucur
	EC-5-glucur	EC-5-glucur
	3'OMeC-5-glucur	3'OMeC-5-glucur
	3'OMeEC-5-glucur	3'OMeEC-5-glucur
Flavonol		Q-glucur (position unknown)
	Q-3-glucur	Q-3-glucur
	MeO-Q-glucur	MeO-Q-glucur
Anthocyanin	Mv-glucoside	Mv-glucoside
	Pt-glucoside	Pt-glucoside
	Dp-glucoside	Dp-glucoside
	Pn-glucoside	Pn-glucoside
	Cy-glucoside	Cy-glucoside
Resveratrol	Res-3-glucur	Res-3-glucur

Plasma Pharmacokinetics of SGP polyphenol metabolites in ZDF versus LN Rats

Eight-hour plasma pharmacokinetic curves of flavan-3-ol metabolites in ZDF and LN rats after 10 days of SGP treatment are shown in Figure 4.6. Plasma levels of four major flavan-3-ol metabolites, C-5-glucur, EC-5-glucur and their methylated metabolites peaked between 1.5 to 2.5 h for ZDF rats and their LN counterparts. This peak was followed by a drop at 4 h for LN rats returning close to baseline by 8h post gavage. ZDF rats exhibited a slower rate of elimination and return to baseline levels. ZDF rats

exhibited a significantly diminished C_{max} compared to their LN respectively for C-5-glucur (p=0.0104), EC-5-glucur (p=0.0077), and their methylated metabolites (p<0.0001) (Table 4.3.).

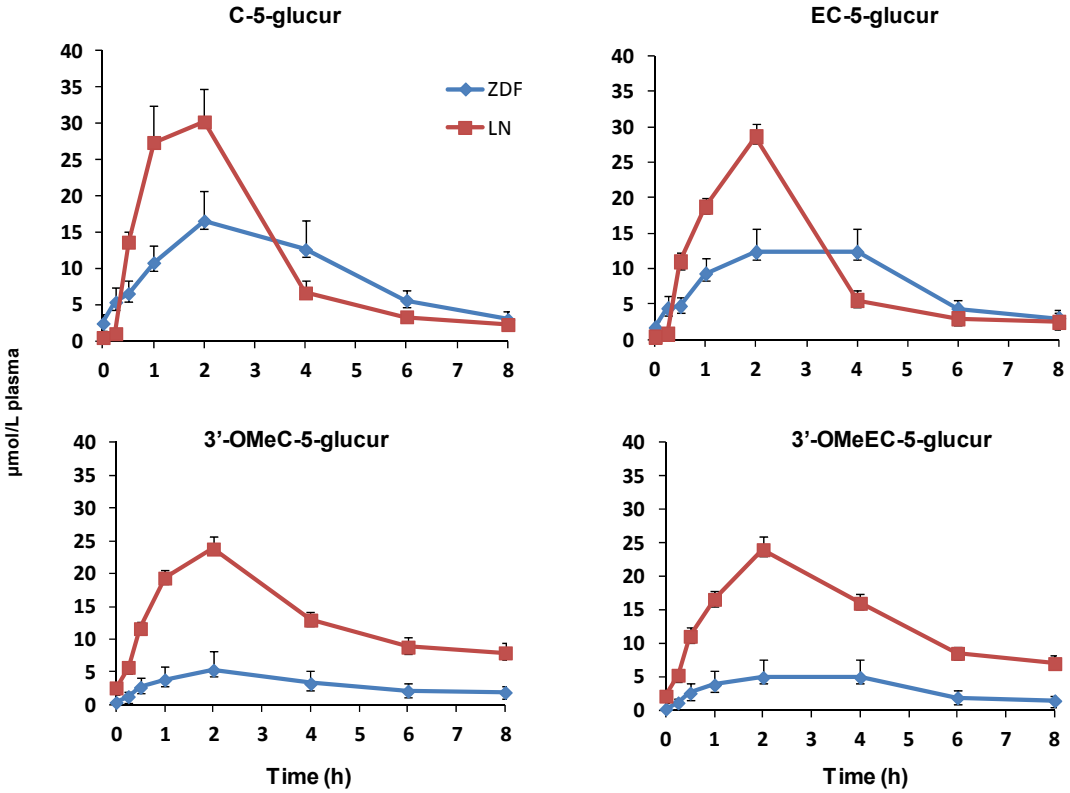


Figure 4.6. Plasma pharmacokinetic responses of flavan-3-ol metabolites.
No metabolites were detected in any control rats (n=4 /CNTL group).
Data was represented as mean ± SEM (n=8 /SGP group).

Table 4.3. Plasma pharmacokinetic parameters of flavan-3-ol metabolites.

		Parameters		
	Treatment	**AUC_{0-8h} (µmol/L*h)**	**C_{max} (µmol/L)**	**T_{max} (h)**
C-5-glucur	ZDF-SGP	76.3 ± 16.7	18.5 ± 3.6	2.3 ± 0.5
	LN-SGP	93.4 ± 11.0	35.9 ± 4.7*	1.5 ± 0.2
EC-5-glucur	ZDF-SGP	59.7 ± 13.5	15.5 ± 2.8	2.1 ± 0.4
	LN-SGP	81.2 ± 11.3	31.3 ± 4.3*	1.6 ± 0.2
3'-OMeC-5-glucur	ZDF-SGP	25.1 ± 13.5	5.4 ± 2.9	1.8 ± 0.2
	LN-SGP	108.1 ± 6.7*	24.8 ± 1.6*	1.8 ± 0.2
3'-OMeEC-5-glucur	ZDF-SGP	23.0 ± 12.4	4.9 ± 2.6	2.0 ± 0.0
	LN-SGP	110.3 ± 5.9*	24.1 ± 1.8*	1.9 ± 0.1

Data was represented as mean ± SEM (n=4 /CNTL group and n=8 /SGP group).
'*' indicated significant difference between ZDF and LN rats with $p<0.05$.

Two major flavonol metabolites derived from Concord grape juice were Q-3-glucur and its methylated glucuronide. Plasma level peaked ~ 1 h for Q-3-glucur at a C_{max} of 8.11 ± 1.42 nmol/L for ZDF rats significantly lower than the C_{max} of 15.41 ± 2.06 nmol/L for their LN counterparts (Figure 4.7.; Table 4.4.). LN rats had MeO-Q-glucur peak before 1h whereas ZDF rats peaked between 2 to 4 h. However, both rodent models had the same residual levels in circulation at 8 h post gavage. ZDF rats reached a C_{max} at 14.29 ± 0.57 nmol/L which was 49% lower than the LN rats (Table 4.4.). This reduction was significant for Q-3-glucur ($p=0.0112$) and for MeO-Q-glucur ($p=0.0062$).

Res-3-glucur plasma levels peaked between 2 to 4 h (Figure 4.7.) reaching a C_{max} of 60.36 ± 6.32 µmol/L for ZDF rats. As with other phenolics, this level was significantly lower than levels observed in LN counterparts with a C_{max} of 34.36 ± 3.48 µmol/L ($p=0.0029$) (Table 4.4.). After peaking, plasma levels in all rats gradully declined but were not completely eliminated by 8 h after gavage. There was difference in the pattern of elimination between ZDF and LN rats. LN rats showed a rapid decline in plasma

concentration after the peak, while ZDF had a more gradual one. However, this difference is not significant based on the values of elimination rate constant (ZDF vs. LN, 0.00 ± 0.04 vs. 0.18 ± 0.20 h^{-1}) .

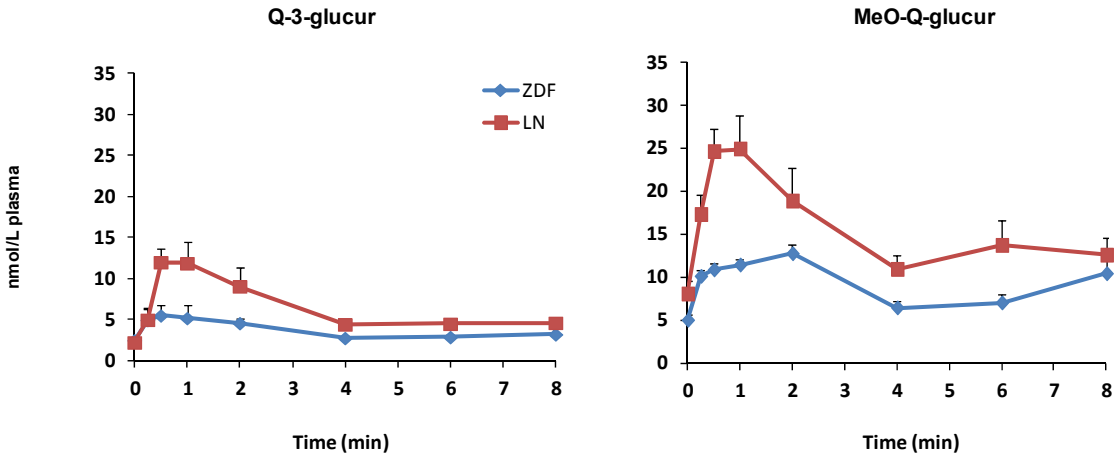


Figure 4.7. Plasma pharmacokinetic responses of quercetin metabolites. No metabolites were detected in any control rats (n=4/CNTL group).
Data was represented as mean ± SEM (n=8/SGP group).

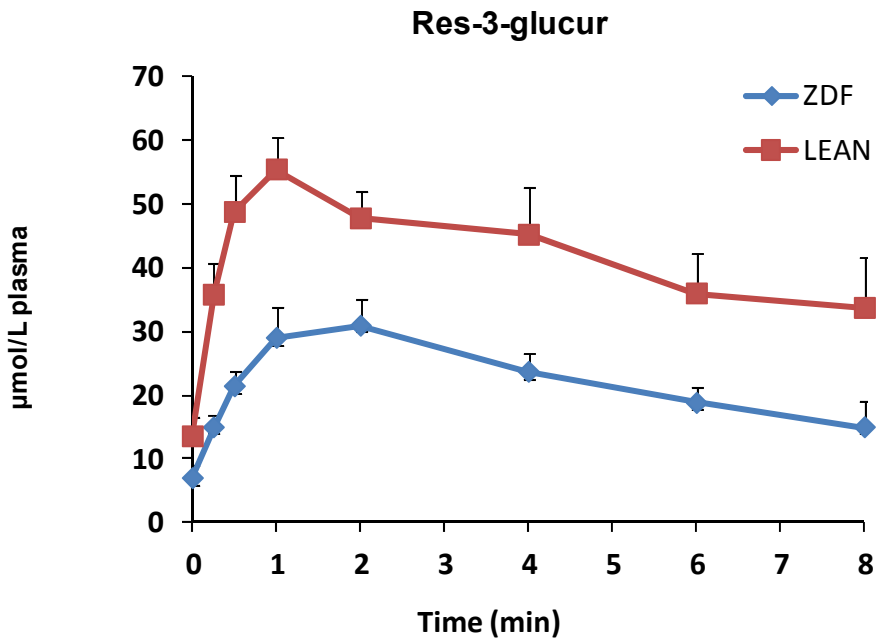


Figure 4.8. Plasma pharmacokinetic responses of resveratrol metabolites. No metabolites were detected in any control rats (n=4/CNTL group).
Data was represented as mean ± SEM (n=8/SGP group).

Table 4.4. Plasma pharmacokinetic parameters of quercetin metabolites.

		Parameters		
	Treatment	**$AUC_{0\text{-}8h}$ (nmol/L*h)**	**C_{max} (nmol/L)**	**T_{max} (h)**
Q-3-glucur	ZDF-SGP	28.6 ± 2.7	8.2 ± 1.4	0.6 ± 0.1
	LN-SGP	50.7 ± 7.3*	15.4 ± 2.1*	1.3 ± 0.4
MeO-Q-glucur	ZDF-SGP	71.9 ± 3.2	14.3 ± 0.6	2.5 ± 0.8
	LN-SGP	123.0 ± 19.9*	27.8 ± 3.5*	0.8 ± 0.2

Data was represented as mean ± SEM (n=4/CNTL group and n=8/SGP group).
'*' indicated significant difference between ZDF and LN rats with $p<0.05$.

Table 4.5. Plasma pharmacokinetic parameters of resveratrol metabolites.

		Parameters		
	Treatment	**$AUC_{0\text{-}8h}$ (μmol/L*h)**	**C_{max} (μmol/L)**	**T_{max} (h)**
Res-3-glucur	ZDF-SGP	180.8 ± 20.5	34.4 ± 3.5	2.6 ± 0.8
	LN-SGP	328.9 ± 40.5*	60.4 ± 6.3*	2.2 ± 0.9

Data was represented as mean ± SEM (n=4/CNTL group and n=8/SGP group).
'*' indicated significant difference between ZDF and LN rats with p<0.05.

Bioavalability, defined as $AUC_{0\text{-}8h}$, were significantly lower in ZDF rats than in LN, for 3'-OMeC-5-glucur (p<0.0001), 3'-OMeEC-5-glucur (p<0.0001) (Table 4.3.). In general, $AUC_{0\text{-}8h}$ of 3'-OMeC-5-glucur, 3'-OMeEC-5-glucur and RES-3-glucur were ~77%, 79% and 45% decreased relative to the LN. Q-3-glucur (p=0.0193), MeO-Q-glucur (p=0.0368) and Res-3-glucur(p=0.0057) were also significantly lower in ZDF rats than LN (Table 4.4. and 4.5.). This suggests impaired bioavailability for ZDF rats relative to the LN rats under the conditions of the present study. Although C-5-glucur and EC-5-glucur did not demonstrate significant differences from their LN counterparts (p=0.0969 and p= 0.0621), the concentrations still showed a diminished trend by 18% and 26% relative to the LN rats.

Anthocyanin plasma profiles indicated a rapid rise between 0.25 to 1 h after SGP administration for LN rats while maximum values were obtained between 0.25 to 1 h for ZDF rats (Figure 4.8.). After reaching peak levels in plasma, Mv-3-glucoside, Pn-glucoside and Cy-3-glucoside concentrations gradually returned to baseline within 8 h measurement in both groups. However, Dp-glucoside exhibited a oblivious second peak around 1 h for both groups possibly resulting from Dp-glucoside being captured again

leaving from intestinal epithelial cell into the portal circulation (Kay 2006). There were no significant differences found between ZDF and their LN counterparts in $AUC_{0\text{-}8h}$, C_{max} or T_{max} for anthocyanins (Table 4.5.). Although it did not reach the criteria for significance, there was a trend of delayed T_{max} of 0.83 ± 0.58 h from ZDF rats on Mv-3-glucoside, Pt-glucoside and Cy-3-glucoside compared to 0.25 ± 0.00 h from LN rats. The delayed T_{max} suggested slower gastrointestinal mobility may be an issue within this diabetic model and merits further exploration.

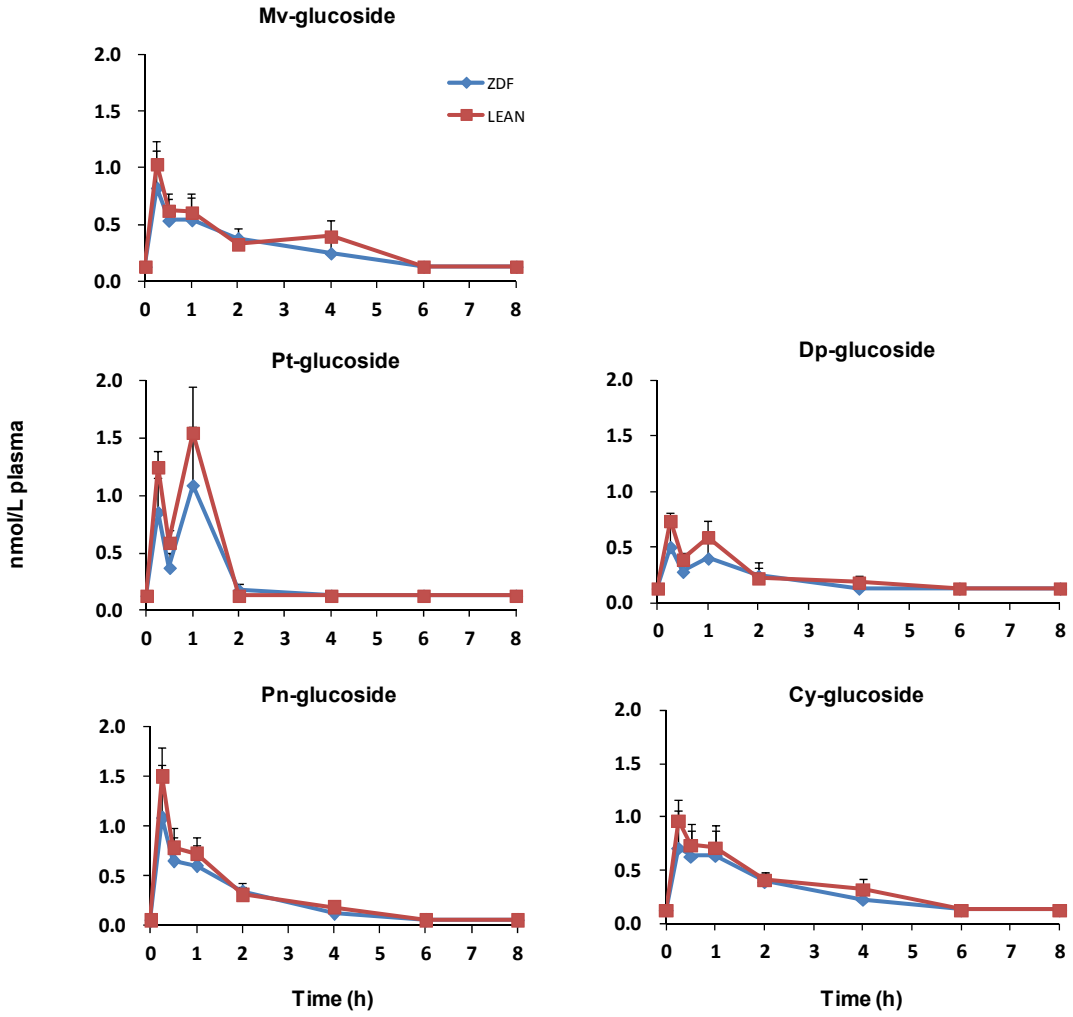


Figure 4.9. Plasma pharmacokinetic responses of key anthocyanins.
No metabolites were detected in any control rats (n=2 with two rats combined /CNTL group).
Data was represented as mean ± SEM (n=3 with two rats combined /SGP group).

Table 4.6. Plasma pharmacokinetic parameters of anthocyanins.

		Parameters		
	Treatment	**$AUC_{0\text{-}8h}$ (nmol/L*h)**	**C_{max} (nmol/L)**	**T_{max} (h)**
Mv-3-glucoside	ZDF-SGP	2.3 ± 0.5	0.9 ± 0.3	0.8 ± 0.6
	LN-SGP	2.7 ± 0.5	1.0 ± 0.2	0.3 ± 0.0
Pt-glucoside	ZDF-SGP	1.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.6
	LN-SGP	1.9 ± 0.3	0.7 ± 0.1	0.3 ± 0.0
Dp-glucoside	ZDF-SGP	2.1 ± 0.4	1.3 ± 0.5	0.8 ± 0.3
	LN-SGP	2.6 ± 0.3	1.7 ± 0.2	0.8 ± 0.3
Pn-glucoside	ZDF-SGP	2.4 ± 0.6	0.8 ± 0.3	0.3 ± 0.0
	LN-SGP	2.8 ± 0.6	1.0 ± 0.2	0.3 ± 0.0
Cy-3-glucoside	ZDF-SGP	1.9 ± 0.5	1.2 ± 0.5	0.8 ± 0.6
	LN-SGP	2.2 ± 0.5	1.5 ± 0.3	0.3 ± 0.0

Data was represented as mean ± SEM (n=2/CNTL group and n=3/SGP group).

Brain Distribution of SGP Polyphenols in ZDF versus LN Rats

There were no polyphenolic compounds detected in any control rats. There were no anthocyanins found in brain tissues of SGP treated ZDF or LN rats. Considering we and others have previously reported anthocyanins in brain tissues (Andres-Lacueva, Shukitt-Hale et al. 2005; Passamonti, Vrhovsek et al. 2005; Talavera, Felgines et al. 2005; Kalt, Blumberg et al. 2008; Milbury and Kalt 2010; Chen, Lobo et al. 2011), this observation suggested that the brain levels were simply below the limit of detection (estimated at 0.42 pmol/g tissue for Mv-glucoside and 0.82 pmol/g tissue for Cy-glucoside). Deposition levels of all other polyhphenols were at pmol/g range after dosing SGP for 10 days. Consistent with plasma data, ZDF rats exibited significantly lower brain concentrations in flavan-3-ol, flavonol and resveratrol metabolites relative to LN counterparts (Figure 4.9.). After nomalizing brain concentrations to SGP dose, brain concentrations of all polyphenol metabolites from SGP were still greater in LN rats

compared to ZDF rats (Table 4.6.). ZDF rats showed significantly lower brain concentrations on C-5-glucur (p=0.0056), EC-5-glucur (p=0.0082), 3'-OMeC-5-glucur (p=0.0114), 3'-OMeEC-5-glucur (p=0.002) and Res-3-glucur (p=0.0088) relative to LN animals. Similarly, brain concentrations of MeO-Q-glucur (p=0.0009) and Q-3-glucur (p=0.0267) for ZDF rats were significantly lower relative to their LN counterparts (Figure 4.9.). This data suggested that diabetic condition in ZDF rats negatively impact the overall absorption and eventual brain deposition on all major grape polyphenol metabolites. When normalized by SGP dose, all four flavan-3-ol metabolites in LN rats still showed higher brain deposition levels compared to ZDF rats. Similar observation was made on quercetin and resveratrol metabolites. Interestingly, flavan-3-ol metabolites seemed to have preferable assess cross the blood-brain barrier compared to quercetin and resveratrol metabolites In both LN and ZDF rats (Table 4.6.).

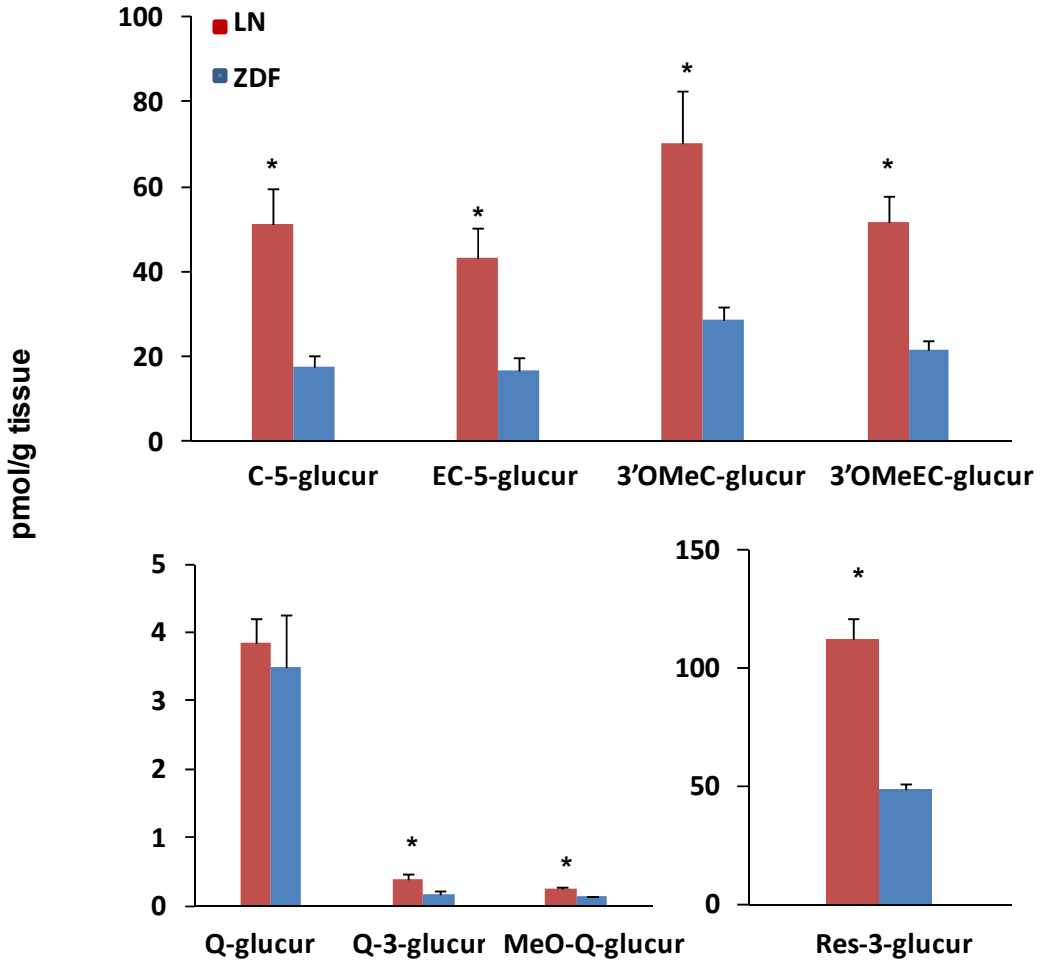


Figure 4.10. Brain responses of flavan-3-ol,resveratrol and quercetin metabolites. No metabolites were detected in control rats.
Data was represented as mean ± SEM (n=4/CNTL group and n=8/SGP group). Presence of asterisk indicates a significant difference between LN and ZDF groups ($p<0.05$).

Table 4.7. Normalized C_{max}, AUC and brain levels by SGP in LN and ZDF rats.

	Normalized by Dose LN rats			Normalized by Dose ZDF rats		
Metabolite	Cmax (μM/mg)	AUC (μM*h/mg)	Brain (nM/mg)	Cmax (μM/mg)	AUC (μM*h/mg)	Brain (nM/mg)
C-5-glucur	12.926	33.625	18.414	6.678	27.474	6.410
EC-5-glucur	9.737	25.235	13.431	4.820	18.553	5.274
3'OMeC-5-glucur	8.914	38.931	25.326	1.934	9.037	10.317
3'OMeEC-5-glucur	7.477	34.266	15.987	1.523	7.132	6.709
Q-3-glucur	0.345	1.137	8.875	0.183	0.642	3.877
MeO-Q-glucur	0.622	2.757	5.827	0.320	1.137	2.913
Mv-glucoside	0.150	0.383	0.000	0.130	0.333	0.000
Pt-glucoside	0.100	0.255	0.000	0.083	0.214	0.000
Dp-glucoside	0.116	0.173	0.000	0.085	0.143	0.000
Pn-glucoside	0.196	0.555	0.000	0.163	0.480	0.000
Cy-glucoside	0.132	0.196	0.000	0.103	0.167	0.000
Res-3-glucur	1.068	5.818	1.988	0.608	3.198	0.865

Urinary Excretion of SGP Polyphenols in ZDF versus LN Rats

Urinary output from ZDF rats was significantly higher with average of 67.39±5.43 mL compared to 4.43±0.32 mL for LN rats. Urinary excretion of polyphenols from SGP was determined based on the sum of polyphenol excretion on day2, day 6 and day10. There were no polyphenols found in baseline urine samples for all rats. After adjusted with total urine volume, sum of each of polyphenol concentration in urine samples were determined (Figure 4.10.). ZDF rats had a significant increase in urinary excretion of C-5-glucur ($p=0.003$), EC-5-glucur ($p<0.0001$) and 3'-OMeC-3-glucur ($p=0.0002$) and 3'OMe-EC-5-glucur ($p=0.0002$). Similar observation was made on Res-3-glucur ($p=0.003$), Q-3-glucur ($p=0.002$) and MeO-Q-glucur ($p=0.04$) that ZDF rats showed significantly higher urinary excretion than LN rats. Anthocyanin excretion was significantly higher in ZDF rats compared to LN rats with Mv-glucoside ($p=0.01$), Pt-glucoside ($p=0.02$), Dp-glucoside ($p=0.001$), Pn-glucoside ($p=0.002$) and Cy-glucoside ($p=0.006$).

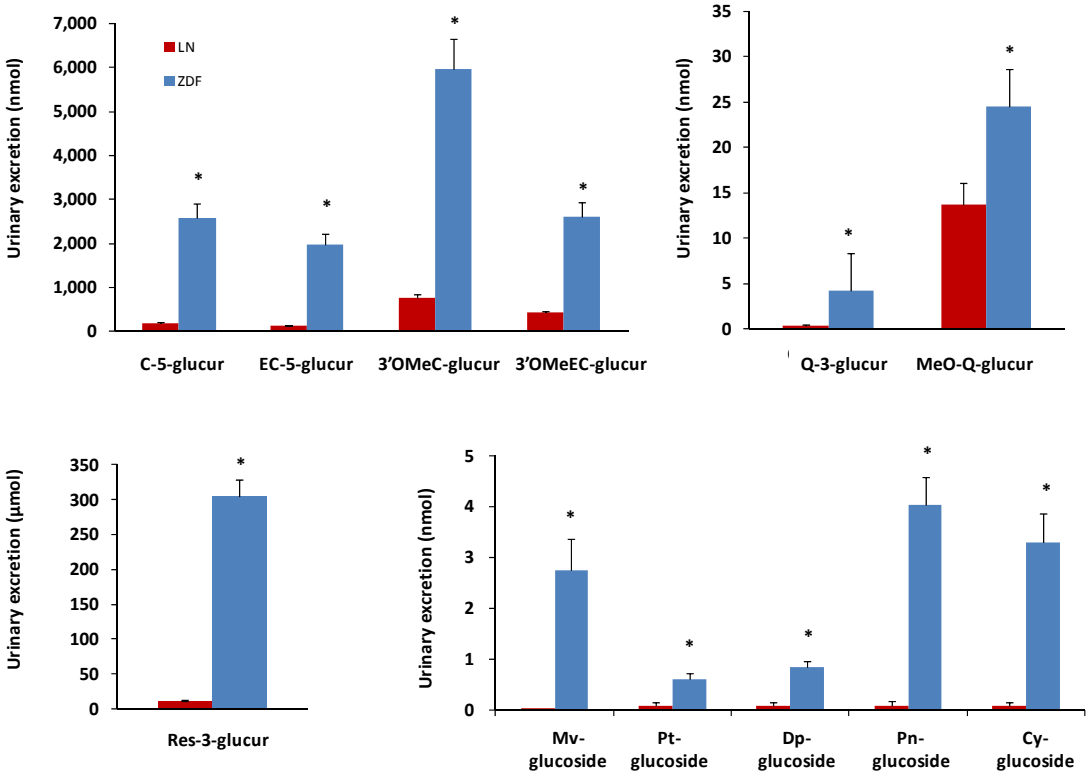


Figure 4.11. Urinary excretion of flavan-3-ol, resveratrol, quercetin metabolites and anthocyanins.
Urine samples were collected from day 2, day 6, day 10 of the study.
Presence of asterisk indicates a significant difference between LN and ZDF groups ($p<0.05$).
Data was represented as mean ± SEM (n=5-8/SGP group).

4.4 Discussion

In general, repeated SGP dosing for 10 days did not influence average body weight gain, food intake and fasting blood glucose for ZDF or LN rats suggesting that SGP is safe for repeated consumption.

The metabolite profiling on polyphenols from SGP was consistent with our previous findings (Ferruzzi, Lobo et al. 2009; Vingtdeux, Giliberto et al. 2010; Ho, Ferruzzi et al.

2012; Wang, Ferruzzi et al. 2012) in Sprague-Dawley (SD) rats treated individually with GSE, red wine or resveratrol suggesting that similar metabolites are found in both SD and ZDF rodent models (Table 4.2.). Furthermore, the consistent metabolite profiles also suggested that SGP treatment may not alter the individual polyphenol metabolism when they were ingested simultaneously as a mixture (discussed in Chapter 3). While this observation warrants future efficacy study in combining proven effective polyphenols for prevention/therapy of AD, it nevertheless suggest that combination of polyphenol sources in extract form may be viable delivery systems.

ZDF rats demonstrated lower plasma levels on major polyphenols from SGP. There is a possibility that the lower bioavailability exhibited in ZDF-SGP rats resulted from heavier in body weight and therefore more blood volume. Polyphenol metabolites present in plasma might be diluted with larger quantity of blood. Therefore, we corrected $AUC_{0\text{-}8h}$ values to total blood volume according to the formula based on body weights for rats (Lee and Blaufox 1985). The results showed that the significance between ZDF-SGP and LN-SGP groups remained the same after corrected for blood volume suggesting that bioavailability of polyphenol metabolites were not diluted by total blood volume in the system.

It has been established that delayed gastric emptying is one of the GI complication resulting from diabetic condition. The condition can be progressed to a clinical disorder of gastroparesis due to complete loss of mechanical outlet that initiate GI muscle movement (Stevens, Jones et al. 2013). It is interesting we observed that ZDF rats

showed a trend of delayed T_{max} on Mv-3-glucoside, Pt-glucoside and Cy-3-glucoside compared to that on LN rats. The delayed T_{max} suggested delayed GI emptying which has been demonstrated in type I and II diabetes models. For example, in a type I rodent model, GI mobility delay was associated with loss of myenteric nitric oxide synthase expression which negatively influenced the function of myenteric plexus (Demedts, Masaoka et al. 2013). Studies investigated compounds or nutrients that may help recover gastric emptying in type II diabetes rodents (Wu, Bound et al. 2013) and humans (Seimon, Brennan et al. 2013) suggesting that it is a problem that needs cure. Based on the knowledge that delayed gastric emptying is a major complication resulted from diabetes, our data show for the first time that polyphenols, especially anthocyanins, showed delayed T_{max} presumably affected by such complication. Therefore, it can be concluded that delayed gastric emptying is a contributing factor for poor polyphenol absorption on diabetic animals, however, as anthocyanins can be effectively absorbed in the gastric environment, delayed emptying was not a hindrance to the uptake of these polyphenols.

Based on the results gathered from plasma data, plasma concentration of all polyphenols including flavan-3-ols, resveratrol, quercetin metabolites and anthocyanins are significantly lower than that in LN rats. It is reasonable to suspect that the low plasma level results from low absorption or high urinary excretion. Our data on urinary excretion confirmed that ZDF rats exhibiting high urinary excretion might be a major factor that influence polyphenol absorption. Further investigation should be focus on

intestinal mechanism of absorption and intestinal integrity and function from SGP of ZDF and LN rats to fully explain the mechanism of the effect of diabetes on polyphenol absorption.

Similar to the speculation we made in Chapter III that flavan-3-ol metabolites are preferably selected to cross blood-brain barrier and enter the brain after normalizing brain concentrations to SGP dose in SD rats (see Chapter 3, Table 3.8.). The normalized data shown in Table 4.6. pointed out similar observation that flavan-3-ol metabolites enter the brain more efficiently compared to the other metabolites in a diabetic rat model. Additionally, when comparing brain levels of major SGP metabolites, LN rats showed higher brain concentrations on all SGP polyphenol metabolites compared to ZDF rats after dose normalization. It has been suggested that the diabetic condition might lead to morphological changes in blood-brain barrier which further influences its functionality. For example, it has been shown that diabetic state induced by streptozotocin in rats had an increase in BBB permeability indicating a microvascular leakage especially to small molecules (Huber, VanGilder et al. 2006). Another study using MR (magnetic resonance) imaging showed an increase in BBB permeability in type II diabetes patients (Starr, Wardlaw et al. 2003). BBB leakage in relation to type II diabetes might explain low concentration of polyphenol metabolites in ZDF brain tissues compared to LN brains presented in the present study. Since many neurodegenerative diseases have been shown to have disrupted BBB structure which lead to functionality breakdown (Ariga, Jarvis et al. 1998; Zlokovic 2002), the BBB leakage that results in low

accumulation of polyphenol metabolites needs to be taken into account when considering the proper usage of dose.

4.5 Conclusion

The goal of this study was to investigate the effect of diabetes on polyphenol bioavailability. To our knowledge, this is the first study that has been conducted in a diabetic rat model to investigate plasma and brain bioavailability of grape polyphenols and their metabolites associated with AD modifying activities. Overall, ZDF rats had diminished plasma bioavailability and brain deposition levels relative to their LN counterparts. ZDF rats showed total urinary excretion on all polyphenols from SGP which inversely correlated to plasma concentration. Connecting plasma and urine data, it is reasonable to speculate that diabetes impaired SGP polyphenol absorption due to excessive excretion from urine. Data from this study support the hypothesis that diabetic condition may have a profound impact on the absorption, metabolism and excretion of grape polyphenols. The mechanism behind these observations merits further investigation including possible alteration of transport and intestinal function as well as major phase II metabolizing enzymes that involve in grape polyphenols transformation. The future direction will be to analyze the level of the same metabolites from gastrointestinal contents as well as the intestines. This will further confirm whether the diminished absorption observed here is due to differences in intestinal transport or processing of polyphenols. With this data implicated, the dosage intended to reach therapeutic efficacy should be adjusted to the disease condition.

CHAPTER 5. PLASMA AND REGIONAL BRAIN BIOAVAILABILITY OF POLYPHENOLS FROM APPLE, GRAPE SEED AND BILBERRY EXTRACTS IN A YOUNG SWINE MODEL

Abstract

Plant derived polyphenols have demonstrated the potential for neuroprotective activities that may influence cognitive development and protection in both young and aging brains. The goal of this project was to determine the bioavailability and regional brain distribution of select polyphenols, including flavan-3-ol, flavonols and anthocynins from apple/grape seed (GSE) and bilberry extracts. Weaning piglets were used as the model of young brains. Animals were treated daily for 3 weeks with two polyphenol-rich products, an apple/GSE blend and a bilberry extract, in a physiological (27.5 mg of total polyphenols/kg BW) and a pharmacological (82.5mg of total polyphenols/kg BW) dose or with water as control. A 24 hour pharmacokinetic study was conducted and brain tissues were harvested and segmented. LC-MS/MS analysis confirmed presence of major flavan-3-ol and flavonol metabolites including catechin-5-glucuronide (C-5-glucur), epicatechin-5-glucuronide (EC-5-glucur), 3'methyl-catechin-5-glucuronide (3'-OMeC-5-glucur), 3'methyl-epicatechin-5-glucuronide (3'-OMeEC-5-glucur), quercetin-glucuronide (Q-glucur) and methyl-quercetin-glucuronide (MeO-Q-glucur) in plasma and regional brain segments from apple/GSE treated groups. Ten key anthocyanindin-galactosides and -glucosides were detected in plasma and brain extracts from bilberry treated groups.

Significant dose-dependent increase was observed in plasma indicated by significantly higher AUC in the high dose apple/GSE group on major flavan-3-ol metabolites. $AUC_{0\text{-}1440min}$ in the unit of μmol/L*min for high versus low apple/GSE groups are 27051 versus 8273 for C-5-glucuronide (p=0.01), 25282 versus 7887 for EC-5-glucuronide (p=0.006), 9662 versus 399 for 3'-OMeC-5-glucuronide (p=0.0004) and 3609 versus 158 for 3'-OMeEC-5-glucuronide (p=0.0007). Most anthocyanidin glycosides exhibit dose-dependent increase in the high dose bilberry group including delphinidin (Dp)- (p=0.04), cyanidin (Cy)-galactosides (p=0.02) and petunidin (Pt)- (p=0.03), peonidin (Pn)- (p=0.03) and cyanidin (Cy)-glucosides (p=0.01). In the brain, significant dose-dependent increase was found in the cerebellum and frontal cortex in all major flavan-3-ol metabolites. All anthocyanidin glycosides except for delphinidin, show a dose-dependent increase in the cerebellum.

5.1 Introduction

There is considerable evidence for the benefits of polyphenol-rich foods and dietary supplements in the prevention of age-related neurodegenerative diseases (see review in(Ebrahimi and Schluesener 2012)). While specific mechanisms remain elusive, it has been proposed that protection against age-related diseases by polyphenols may be due, in part, to their antioxidant properties, their potential to increase of nitric oxide production and through anti-inflammatory mechanism (Campos-Esparza Mdel and Torres-Ramos 2010; Albarracin, Stab et al. 2012). There is also a growing interest in the

potential of polyphenols to promote healthy brain and cognitive development in young population. However, there are few studies of the benefits of polyphenols in young populations. Most investigations have focused on the prevention of injury in prenatal life. For example, Loren et al. showed that maternal supplementation with pomegranate juice protected hypoxic-ischemic injury in rat pup's brain (Loren, Seeram et al. 2005). Narita et al. demonstrated that grape seed extract protected glutamate-induced insults in a primary neonatal mouse neuron culture (Narita, Hisamoto et al. 2011).

A key step in understanding the potential of polyphenols to promote healthy brain development is the determination of dose response, bioavailability, metabolism and tissue distribution of specific polyphenols and metabolites. However, limited data exists on the estimation of polyphenol intake on infants and children. As far as polyphenols intake in the young population, Gu et al. estimated ~68 mg/day of proanthocyanidins intake in the age group of 2-5 years old, ~65mg/day in 6-11 years old, ~1.3mg/day in infants (4-6 month) and ~26.9 mg/day in 6-10 month in the United States (Gu, Kelm et al. 2004). In an Australian population, total flavonoid intakes were estimated to be ~24, ~28 and ~40 mg/day in children in age groups 2-3, 4-7 and 8-11 years old (Johannot and Somerset 2006). A recent study estimated anthocyanidin intake of ~4mg/day (median) in older infants and toddlers aged 9-36 month old (Drossard, Alexy et al. 2011). Based on these three studies, an estimated intake of flavonoids would be <100 mg/day in infants and children. In this study, we used polyphenol-rich apple/GSE and bilberry

extracts, which are abundant in major flavonoids and anthocyanin glycosides was appropriate for targeting infants and young children consumption.

Understanding of bioavailability, metabolism and tissue distribution of polyphenols from fruits and vegetables is critical since the physiological impacts of polyphenols depend on their delivery to target tissues. Previous investigations have identified key dietary polyphenol forms that have the potential of penetrating the blood-brain barrier in animal models and whose metabolites may elicit neuroprotective effects (Vingtdeux, Giliberto et al. 2010; Wang, Ferruzzi et al. 2012; Ho, Ferruzzi et al. 2013). This includes flavonoids such as flavan-3-ols, flavonols and anthocyanidins derived from fruits. Apples are highly abundant in flavonols especially quercetin derivatives including Q-3-galactoside and Q-3-glucoside (Lee, Kim et al. 2003). Grape seeds are rich in flavan-3-ols primarily monomeric catechin (C) and epicatechin (EC) and polymeric procyanidins (Hollecker, Pinna et al. 2009). Most berries are rich in anthocyanindin sugar derivatives such as glucosides, galactosides and arabinosides (Latti, Riihinen et al. 2011; Liang, Yang et al. 2012). A number of studies report bioavailability of these specific flavonoids from different food stuffs in various species including humans (see review in (Scalbert and Williamson 2000; Manach, Williamson et al. 2005)), rats (Nakagawa and Miyazawa 1997; Abd El Mohsen, Kuhnle et al. 2002; Ferruzzi, Lobo et al. 2009; Prasain, Peng et al. 2009; Serra, Macia et al. 2012), and pigs (de Boer, Dihal et al. 2005; Percival 2005; Bieger, Cermak et al. 2008).

To better understand the potential of these polyphenols to deliver neuroprotective benefits in young populations, it is important to determine whether the distribution of polyphenols and metabolites in the brain is uniform or if there are regional differences. However, determination of brain distribution has often not been complete due to analytical limitations and insufficient quantities of tissue. Ferruzzi et al. reported ~290 and ~576 pg/g of C, methyl-catechin (MeO-C) and EC, MeO-EC in whole rat brain after 10 days of intragastric gavage of 50-150mg/kg BW of GSE for 10 days (Ferruzzi, Lobo et al. 2009). Ishisaka et al. found ~40 pmol/g of Q aglycon and ~48 pmol/g of MeO-Q after feeding rats a quercetin-containing diet at the dose of 200mg/kg BW for one month (Ishisaka, Ichikawa et al. 2011). Bieger et al. found~0.02 nmol/g of Q aglycon in pig brains after 4 weeks of quercetin-containing diet consumed at the dose of 50 mg/kg BW (Bieger, Cermak et al. 2008). A feeding trial in which, rats were put on 2% of blueberry powder for 8 weeks resulted in detection of anthocyanidin aglycon in brain regions, however the concentrations were not estimated (Andres-Lacueva, Shukitt-Hale et al. 2005). Milbury and Kalt detected ~0.7 to 0.9 nmol/kg tissue of anthocyanins and glucuronides in brain regions after feeding pigs with 2% of blueberry diet for 8 weeks (Milbury and Kalt 2010). More data is needed to correlate brain deposition of polyphenols to their physiological functions in vivo.

Pigs are suitable model for nutrient studies that provide implications for human nutrition. In spite of some external morphological differences between pigs and human, their anatomy and physiology are similar (Patterson, Lei et al. 2008). The similarity in

gastrointestinal (GI) structures between pigs and humans makes the pig a good model to generate preliminary data for human nutrition studies (Patterson, Lei et al. 2008). Pigs are omnivores like humans with similar absorption and metabolic processes. Besides GI similarity, another advantage to using pigs over rodents for brain distribution studies is the larger amount of brain material. The large available amount of tissue makes it possible to determine differential distribution of polyphenols in different brain regions.

We reported here on data obtained from bioavailability studies from piglets treated with two polyphenol preparations, apple/GSE blend and bilberry extract, in high and low doses. The major goals were: 1) to investigate plasma pharmacokinetic behavior and dose response and 2) to determine regional brain distribution and dose response of polyphenols derived from commonly consumed fruit extracts (apple, grape seed and bilberry). This study provides evidence to suggest that consumption of physiological dose of polyphenols can result in their accumulation in specific brain regions providing as basis for future efficacy studies.

5.2 Materials and Method

Chemicals and Materials

(+)-C, (-)-EC, procyanidin B1, B2 and Q-3-glucuronide authentic standards were purchased from Sigma-Aldrich (St. Louis, MO). All extraction and liquid chromatography solvents were HPLC certified and were obtained from J.T. Baker (Phillipsburg, NJ). Mv-3-

glucoside chloride and Cy-3-glucoside chloride were purchased from ChromaDex (Irvine, CA). Apple extract (Appl'in AFPOMM9080) was purchased from Diana Naturals (Antrain, France) and GSE (OmniVin 20R) was from Ajinomoto OmniChem (Louvain-la-Neuve, Belgium). The apple/GSE blend used in this present study was a mix at 2:1 ratio. Bilberry extract (NutriPhy) was purchased from ChrHansen (Horsholm, Denmark).

Animals

All animal studies were conducted under guidance and with protocols reviewed and approved by the Purdue University Animal Care and Use Committee. Forty commercial, newly weaned piglets with body weight of 14 ± 0.5 pounds were used. After one week of acclimation, piglets were randomly assigned to five treatment groups: 1) control (CNTL), 2) high dose apple/GSE blend (high apple/GSE), 3) low dose apple/GSE blend (low apple/GSE), 4) high dose bilberry extract (high bilberry) and 5) low dose bilberry extract (low bilberry). Piglets were maintained on polyphenol free pelleted diet D01067 for growing swine (Research Diets, New Brunswick, NJ) with casein replacing soy protein providing 308 kcal/g of pellet. In order to control the body weight gain and maintain healthy growth, piglets were fed on 4% of their body weight for the first two weeks and then 5% of their body weight for the remainder of the study (Carr 1998). The median body weight of the pigs was used to calculate the food amount given to all piglets.

Material Analysis

Analyses of extracts and diets was performed based on the method described in (Song, Jouni et al. 2013) for apple/GSE blend and in (Song, Sapper et al. 2013) for bilberry extract. In brief, both analyses were completed by a Waters 2695 Separations Module with a Waters Xterra RP-C18 column (2.1 x 100 mm, 3.5 µm) at 0.3 mL/min flow rate. The column was heated to 40℃ for apple/GSE and 35℃ for bilberry analysis. A Waters ZQ MSD was used to detect phenolics with single ion responses (SIRs) in apple/GSE including 289, 301 and 463 m/z to target C/EC, Q aglycon and Q-3-glucoside, respectively. SIRs for bilberry were set at 303.8, 331.7, 287.5, 317.7 and 301.7 to detect Dp, Mv, Cy, Pt and Pn, respectively. Total polyphenolic content of apple/GSE and bilberry was determined to be 54% and 89% by Folin-Ciocalteu assay.

Polyphenol Treatment

The target dose for young children will be 200 mg/day based previous reports (Gu, Kelm et al. 2004; Johannot and Somerset 2006; Drossard, Alexy et al. 2011). In order to accurately translate data from piglet to children, we adjusted the doses used in this study using standard FDA conversions between animal and human equivalent dose (FDA 2005). The conversion gave a reasonable dose of 27.5 mg/kg BW in a piglet equaled to 200 mg/kg BW in a human child. This dose was estimated to be close to physiological dose achievable by diet. A pharmacological/supplemental dose was designed to be 3 times higher (82.5 mg/kg) to resemble the effect of drug unachievable by food

consumption. The amounts for treatments were calculated based on the total polyphenol content of the two polyphenol-rich preparations. The polyphenol treatments were given every morning mixed with a small amount of food (~50 to 90g depending on body weight) and water for total of three weeks. Piglets in the control group received water mixed with plain food. Supplements were consumed within 2 to 3 minutes. After consuming their supplements, piglets were fed 2/3 of their daily food allowance and the remaining 1/3 was fed in the afternoon.

Pharmacokinetic Study

In the last week of the study, the piglets were implanted with jugular vein catheters (CX-2014S, BASi, West Lafayette, IN) based on the procedure described in (Marchant-Forde, Matthews et al. 2012) with minor modifications. In brief, piglets were anesthetized with intramuscular injection of 0.025 mL/kg body weight of TKX (Telazol, Ketamine and Xylazine) obtained from Lloyd Laboratories, Shenandoah, IA and maintained under anesthesia with 2.5% of isoflurane with 3 L/min of oxygen. An incision was made over the jugular fossa for the catheter placement. After checking for patency, the incisions were closed. Piglets were injected with 1 mg/kg BW of 100 mg/mL Banamine (Prevail^{TM}, VETONE Inc. League City, TX) for post-surgical analgesia. A second dose of Banamine was given 8 h post surgery.

The day after surgery and following an overnight fast, a pharmacokinetic assessment was initiated. A baseline blood sample was obtained and piglets were dosed with

supplements mixing with pelleted diet and water. After individual polyphenol treatments, blood was collected at the following predetermined time intervals: immediately post treatment (~5minutes), 10, 20, 30, 45 minutes and 1, 1.5, 2, 3, 4, 6, 8, 12 and 24h post dose. All blood samples were drawn from the catheter, placed into heparinized tubes and centrifuged at 6500 rpm for 10 min at 4 ℃ to separate plasma. Plasma was collected and acidified with 1% ascorbic acid (w/v) in saline at 4:1 ratio, purged with N_2 and frozen. Food was return to piglets 1h after supplement dosing.

Body Weight, Food Intake and Liver Toxicity

Piglets' body weights were monitored every other day and food intake was measured every day throughout the study to ensure proper growth. After the final 24h blood draw, piglets were dosed with one additional polyphenol treatment. One hour post dosing, blood samples were drawn and serum was obtained. Serum was analyzed for liver enzyme levels using the Kodak Ektachem 700 analyzer (Eastman Kodak Company, Rochester, NY) to check for toxicity. Piglets were then euthanized with 1mL/10lbs BW of Beuthanasia (Intervet/Schering-Plough, UK). Blood was removed from brain tissue by perfusion with ice-cold saline into the descending aorta and heart. Six brain regions including cerebellum (Ce), brain stem (St), medial frontal cortex (Co), hippocampus (Hi), hypothalamus (Hy) and amygdala (Am) were collected within 15 minutes of euthanasia and immediately frozen until analysis.

Polyphenol Metabolites Extraction in Plasma and Brain Tissues

C/EC and Q metabolites and anthocyanin glycosides were extracted from plasma and brain homogenates by solid phase extraction (SPE) using 1 mL Waters Oasis HLB cartridges (Milford, MA) as previously described (Ho, Ferruzzi et al. 2013). In brief, acidified plasma and methanolic brain extracts were loaded onto preconditioned SPE cartridges. The cartridges were washed with 1 mL of 1.5M formic acid (v/v) followed by 1 mL of 5% aqueous methanol (v/v) for C/EC and Q metabolites and 2 mL of 2% formic acid (v/v) for anthocyanidin derivatives. C/EC and Q metabolites were eluted with 2 mL of 0.1% formic acid/methanol (v/v) and anthocyanidin derivatives were eluted with 2% formic acid/methanol (v/v). Eluents were dried under vacuum at 37 ℃. Dried extracts were reconstituted with LC mobile phases for immediate analysis. For the bilberry treated groups, regional brain tissues from two piglets were combined for anthocyanin analysis. Amygdala parts from two piglets in the same apple/GSE group were combined for C/EC and Q analysis.

Polyphenol Analysis of Plasma and Brain Tissues by LC-MS/MS

Polyphenol analyses were performed on an Agilent 6400 Triple Quadrupole LC-MS/MS equipped with an electron spray ionization (ESI) source under multiple reaction monitoring modes (MRM) as previously described in (Wang, Ferruzzi et al. 2012; Ho, Ferruzzi et al. 2013) with minor modifications. A Waters XTerra RP-C18 column (2.1 x 100 mm, 3.5 μm) was employed with binary mobile phases of A: 0.1% aqueous formic

acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v). The column was heated to 30℃ and the system flow rate was 0.3 mL/min. The binary gradient to elute all phenolic metabolites was: 10% B at 0 min, 40% B at 10 min, 95% B at 11 min and back to 10% B at 12 min to 18 min. Fragmentor voltage was set at 135V and collision energy was 17eV for all compounds. ESI source conditions: gas temperature was 350℃, drying gas flow was 11 l/min, nebulizer was 30 psi, sheath gas temp was 350℃, sheath gas flow was 11 L/min, capillary voltage was 3500V and nozzle voltage was 1000V. For anthocyanin analysis, the column was heated to 35℃ and the binary mobile phases were A: 2% aqueous formic acid (v/v) and B: 0.1% formic acid in acetonitrile (v/v). The gradient was: 5% B at 0 min, 10% B at 10 min, 25% B at 30 min, 5% B at 31 min and continue on 5% B to 35 min. ESI source condition setting was the same as described above. MRM transitions of the targeted phenolics are shown in Table 5.1. Quantification of C/EC and Q metabolites was based on MS peaks estimated using calibration curves constructed from authentic C aglycon and Q-3-glucur standards. Quantification of Cy-3-glucoside was calculated from a calibration curve constructed with authentic Cy-3-glucoside while other anthocyanidin glycosides were based on Mv-3-glucoside.

Table 5.1. MRM transition and ESI source polarity for flavan-3-ol, quercetin metabolites and anthocyanidin glycosides.

Identification	MRM Transition	ESI Polarity
(E)C-5-glucur	465.1 -> 289.1	Negative
3'-OMe(E)C-5-glucur	479.1 -> 303.1	Negative
Q-glucur	479.1 -> 303.1	Positive
MeO-Q-glucur	493.1 -> 317.1	Positive
Mv-glucoside/galactoside	493.1 -> 331.1	Positive
Pt-glucoside/galactoside	479.1 -> 317.1	Positive
Dp-glucoside/galactoside	465.1 -> 303.1	Positive
Pn-glucoside/galactoside	463.1 -> 301.1	Positive
Cy-glucoside/galactoside	449.1 -> 287.1	Positive

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Pharmacokinetic parameters including: 1) area under the plasma concentration versus time ($AUC_{0\text{-}1440min}$) were calculated using the linear trapezoidal rule, 2) the maximum plasma concentrations (C_{max}) and, 3) the time at C_{max} (T_{max}) were determined directly from the pharmacokinetic curves of plasma concentration versus time. Statistical analyses were performed using SAS 9.3 statistical analysis program (Cary, NC). Group differences in body weight were analyzed by a repeated measurement model. Group differences on food intake AUC, liver enzyme levels and brain regions within the same dose group were determined by one-way ANOVA with Tukey's post-hoc test. Differences between high

and low dose groups within the same treatment for pharmacokinetic parameters and brain concentrations were analyzed by Student's t test. Non normal distributed data were subjected to transformation with appropriate lambda value. Student's t test were applied to determine the significant difference between transformed data in high and low dose groups. The significance was accepted at the level of $\alpha<0.05$.

5.3 Results

Polyphenol Contents of Treatment Materials

Primary polyphenols detected in 2:1 apple/GSE blend are depicted in Figure 5.1. The major polyphenols found in apple/GSE blend included monomeric C, EC and Q-3-glucoside, Q aglycon with quantities of 21, 40, 16 and 0.93 mg/g of extract respectively. Procyanidin B1 and B2 were determined to be 17 and 32 mg/g of extract. Other polyphenols found in apple/GSE blend are shown with quantities in Table 5.2. Major bilberry extract anthocynidin-glycosides including galactoside, glucoside and arabinoside derivatives were shown in Figure 5.2 and quantities in Table 5.2. The analysis data was provided by Brian Song.

Table 5.2. Quantities of major flavonoids detected in 2:1 apple/GSE blend and major anthocyanidin glycosides detected in bilberry extract.

Identification	2:1 apple/GSE mix Compound (mg) / Extract (g)
Catechin	21.06
Epicatechin	40.24
Epigallocatechin	0.19
Epicatechin Gallate	3.44
Quercetin	0.93
Quercetin-3-glucoside	16.14
Procyanidin B1	17.65
Procyanidin B2	32.27
Other Procyanidin	9.74
Other Procyanidin	1.64

Tentative ID	Bilberry extract Aglycon (mg) / Extract (g)
Pn galactoside	0.79
Pn glucoside	4.71
Pn arabinoside	0.97
Dp galactoside	26.09
Dp glucoside	69.69
Dp arabinoside	19.05
Mv galactoside	2.58
Mv glucoside	8.04
Mv arabinoside	2.52
Pt galactoside	3.53
Pt glucoside	10.58
Pt arabinoside	2.81
Cy galactoside	2.62
Cy glucoside	5.43
Cy arabinoside	3.18

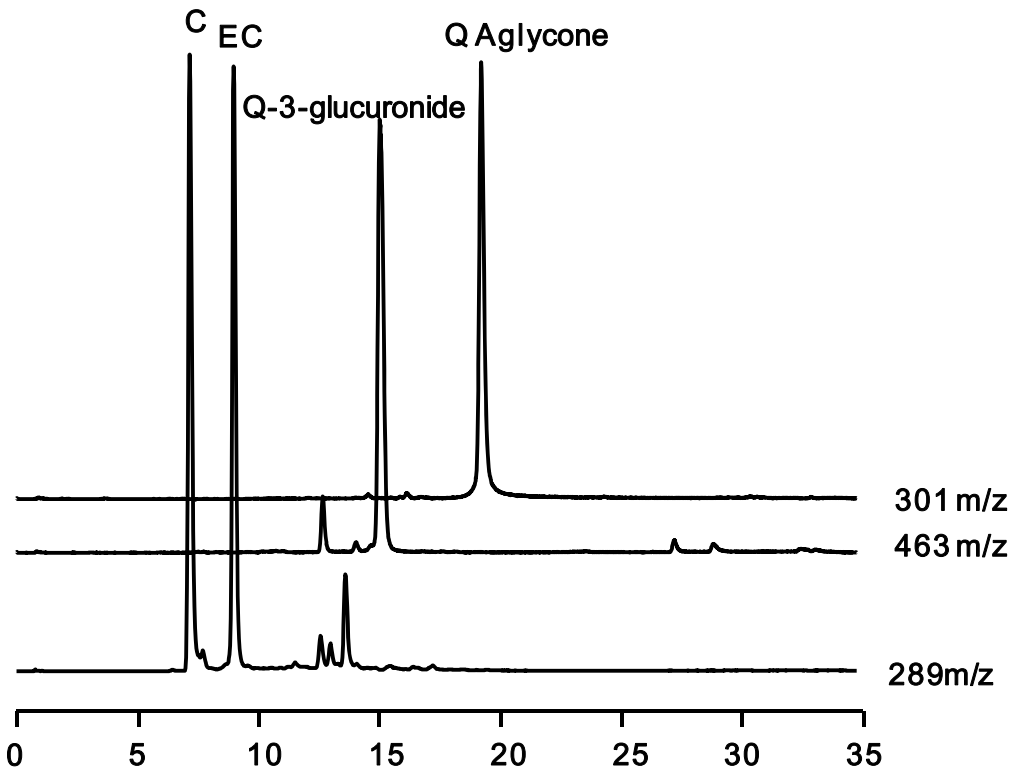


Figure 5.1. LC-MS separation of major polyphenols in 2:1 apple/GSE blend.
LC-MS data shows the presence of C and EC (289m/z), Q-3-glucur (463m/z) and Q aglycon (301m/z).

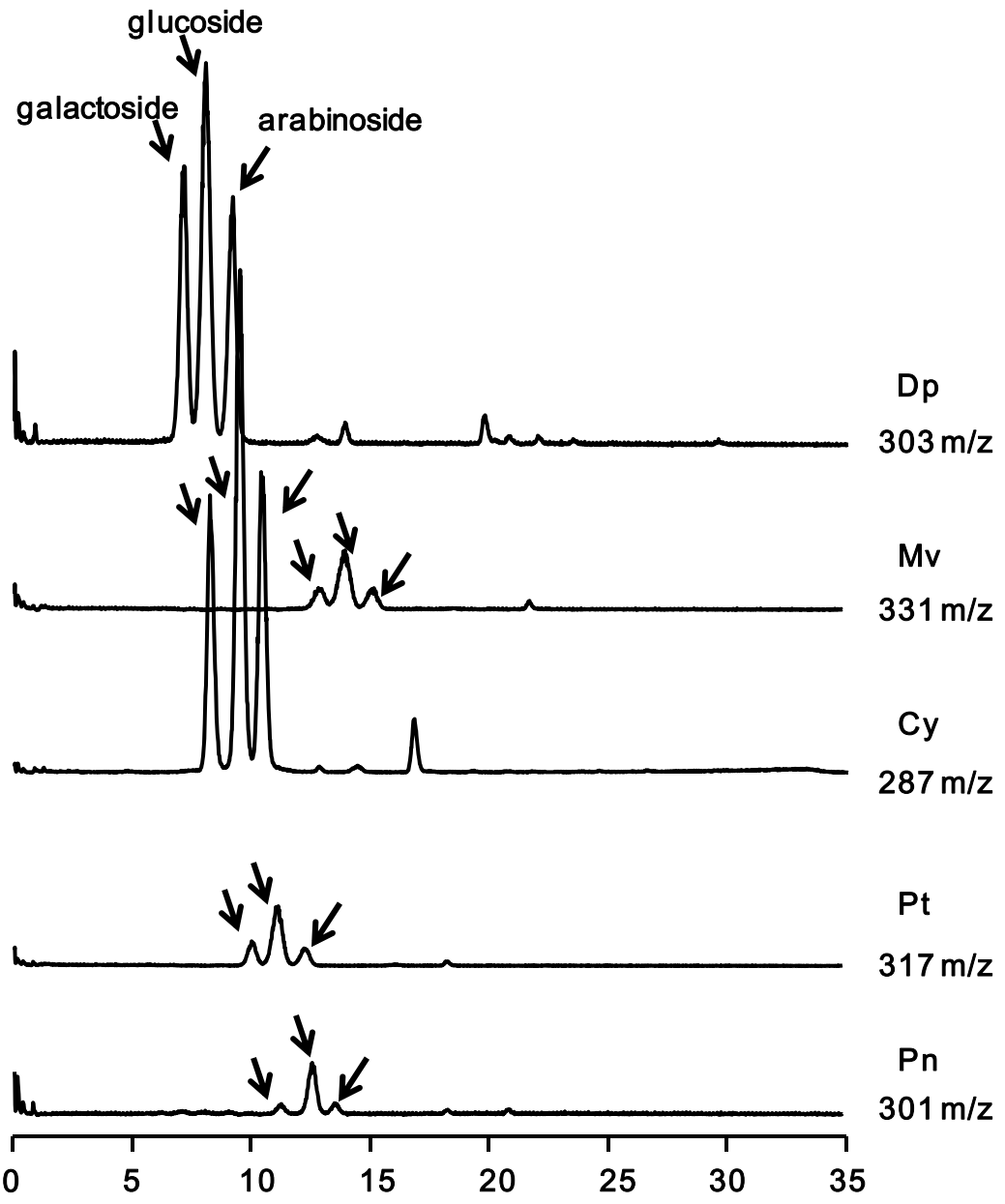


Figure 5.2. LC-MS separation of major anthocyanidin glycosides in bilberry extract in aglycon forms.
LC-MS data shows the presence of sugar derivatives in the order of galactoside, glucoside and arabinoside for Pn (301.7m/z), Pt (317.7m/z), Cy (287.5m/z), Mv (331.7m/z) and Dp (303.8m/z).

Body Weight, Food Intake and Toxicity

The growth of the piglets was monitored by recording both body weight and food intake. There was no significant difference in food intake between groups suggesting that polyphenol treatments did not affect appetite. Body weight did not differ among groups indicating that polyphenol treatments likely did not influence normal growth during the 3-week period of this investigation (data not shown).

Liver enzymes including aspartate aminotransferase (AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) as well as total bilirubin were determined in serum as measures of potential toxicity of each extract (Table 5.3.). Reference ranges were based values for commercial swine (Friendship, Lumsden et al. 1984). AST levels appeared to be within normal range for all groups. The ALP of the high apple/GSE group was slightly higher than the normal range (Table 5.3.). However, there was no statistically difference between treatment groups. All polyphenol treatment groups showed higher levels of GGT than the normal range while the control group was at the high end of the reference range. There was no significant difference among groups in GGT levels.

Table 5.3. Liver enzyme panel of piglets.

Analyte	Reference Range	Treatment	Average	SEM	Compared to ref. range
Total Billirubin	0-0.3	CNTL	0.21	0.02	
	mg/dL	High apple/GSE	0.23	0.03	
		Low apple/GSE	0.23	0.06	
		High Bilberry	0.21	0.03	
		Low Bilberry	0.14	0.04	
Aspartate Aminotransferase	16-65	CNTL	34.13	2.95	
(AST)	IU/L	High apple/GSE	43.60	6.68	
		Low apple/GSE	39.89	4.05	
		High Bilberry	51.27	9.45	
		Low Bilberry	35.38	3.46	
Alkaline Phosphatase	92-294	CNTL	277.50	17.03	
(ALP)	IU/L	High apple/GSE	296.60	33.24	High
		Low apple/GSE	277.11	14.50	
		High Bilberry	262.00	23.56	
		Low Bilberry	276.00	32.05	
γ-Glutamyl Transferase	16-30	CNTL	30.00	2.66	
(GGT)	IU/L	High apple/GSE	34.20	9.26	High
		Low apple/GSE	39.89	6.17	High
		High Bilberry	32.55	3.80	High
		Low Bilberry	31.00	3.66	High

Characterization of major polyphenols in plasma and brain tissues of piglets treated with apple/GSE and bilberry extract

Major flavan-3-ol and flavonol metabolites detected in plasma and brain extracts from apple/GSE treated piglets included C/EC-5-glucur, 3'OMe-C/EC-5-glucur (Figure 5.3.), Q-glucur and MeO-Q-glucur (Figure 5.4.). The results confirmed that the C/EC and Q polyphenols are in fact bioavailable from these extracts and specific metabolites found in plasma can cross blood-brain barrier and accumulate in brain tissue. The assignment of the Q-glucuronide remains to be completed. However, based on comparison with authentic Q-3-glucuronide standard, we can eliminate the glucuronidation at position 3 as the likely structure. MeO-Q-glucuronide was detected in some regional brain extracts but the level was below LOQ (1.70 pg of Q-3-glucuronide on column).

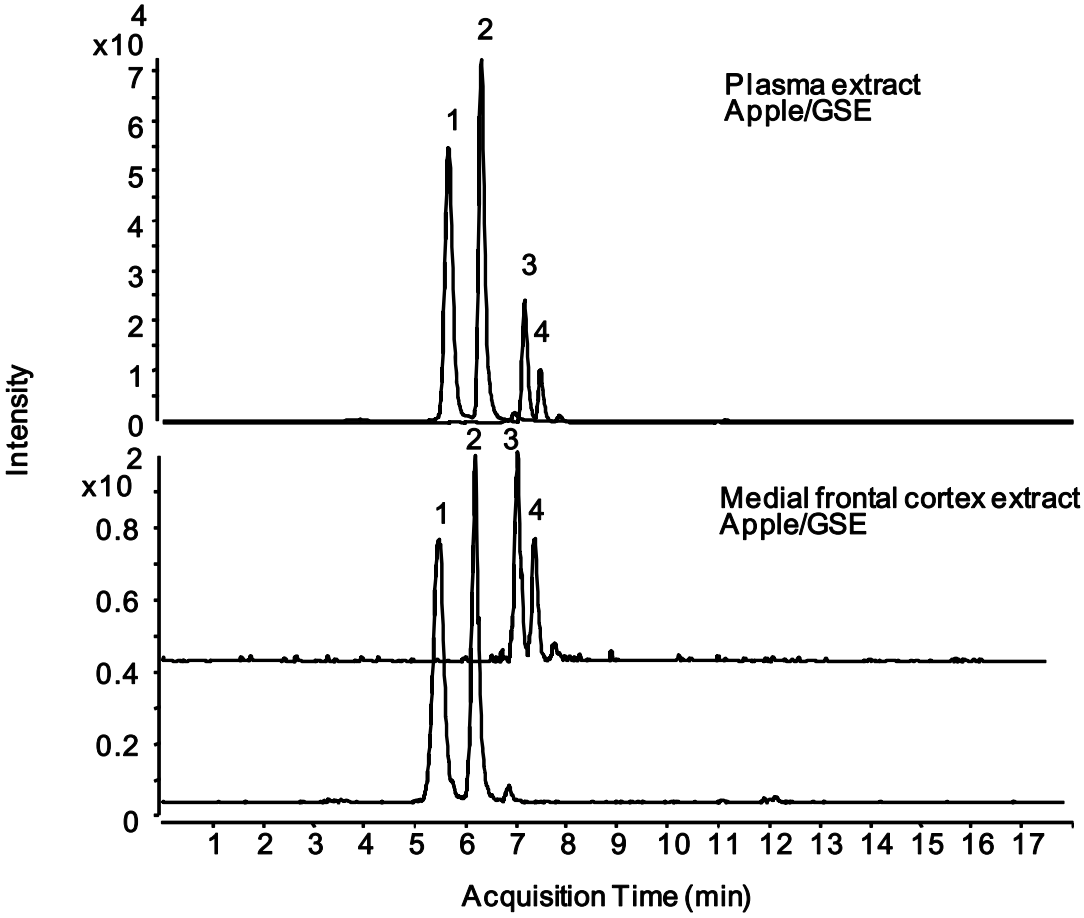


Figure 5.3. Representative MRM chromatograms of flavan-3-ol metabolites in plasma and brain extracts from apple/GSE treated piglets.
MRM chromatogram is shown for C/EC-5-glucur (465.1 -> 289.1 m/z) and 3'OMe-C/EC-5- glucur (479.1 -> 303.1 m/z) under negative polarity. Peaks identifications are: 1. C-5-glucur, 2. EC-5-glucur, 3. 3'-OMeC-glucur and 4. 3'-OMeEC-glucur.

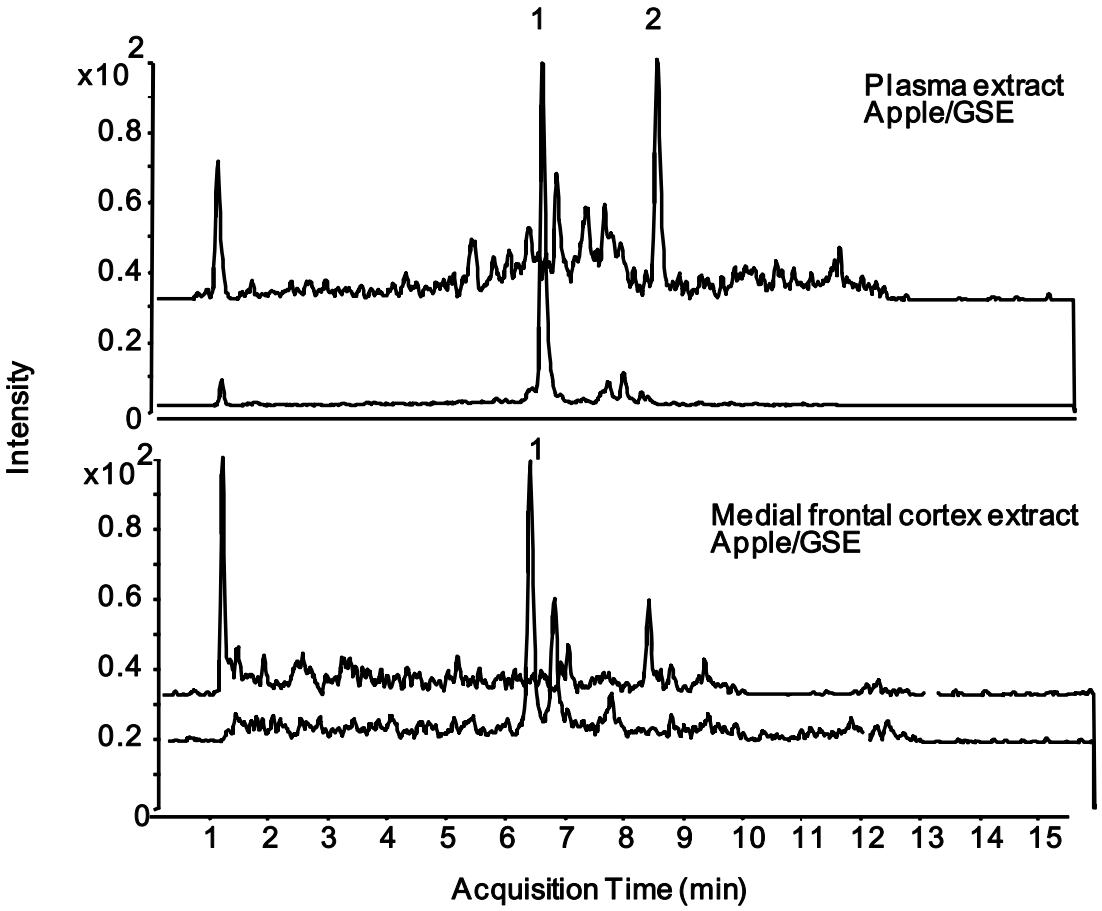


Figure 5.4. Representative MRM chromatograms of major Q metabolites in plasma and brain extracts from apple/GSE treated piglets.
MRM trace is shown for Q-glucur (479.1 -> 303.1 m/z) and MeO-Q-glucur (493.1 -> 317.1 m/z) under positive mode. Peak identifications were: 1. tentatively identified as Q-glucur and 2. MeO-Q-glucur. MeO-Q-glucur was present in some brain extracts but the level was < LOQ.

Ten key anthocyanidin glycosides including Mv-, Pt-, Dp-, Pn, and Cy-galactosides and glucosides were detected in plasma and brain extracts from bilberry treated piglets (Figure 5.5.). Retention time on anthocyanidin-galactosides and anthocyanidin-glucosides with the same MRM transition (Table 5.1.) were tentatively determined based on previous reports (Zhang, Kou et al. 2004; Wu and Prior 2005; Vafeiadou,

Vauzour et al. 2009; Ha, Lee et al. 2010). Based on information from previous reports and validation with authentic standards of Mv- and Cy-3-glucosides, the tentative identification of the peaks were assigned in the order of anthocyanidin-galactosides followed by anthocyanidin-glucosides in plasma and brain extracts from bilberry treated piglets.

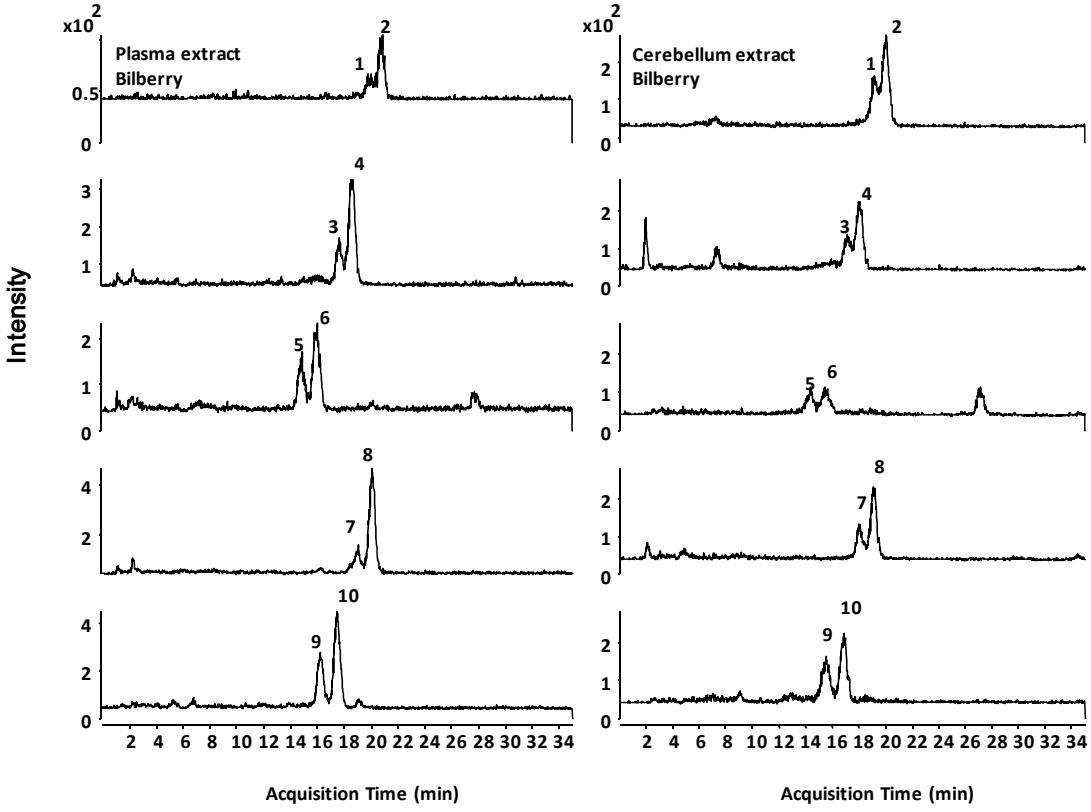


Figure 5.5. Representative MRM chromatograms of anthocyanidin glycosides in plasma and brain extracts from bilberry treated piglets.
MRM transitions were: 493.1 -> 331.1 for Mv-galactoside/glucoside, 479.1 ->317.1 for Pt-galactoside/glucoside, 465.1 -> 303.1 Dp-galactoside/glucoside, 463.1 -> 301.1 for Pn-galactoside/glucoside and 449.1 -> 287.1 for Cy- galactoside/glucoside. Peak identifications were: 1. Mv-3-O-galatoside, 2. Mv-3-O-glucoside, 3. Pt-galactoside, 4. Pt-glucoside, 5. Dp-galactoside, 6. Dp-glucoside, 7. Pn-galactoside, 8. Pn-glucoside, 9. Cy-galactoside and 10. Cy-glucoside.

Dose effect on plasma pharmacokinetics of polyphenols from apple/GSE or bilberry treated piglets

24 hour pharmacokinetic curves of C and EC metabolites are illustrated in Figure 5.6. Plasma reached peak concentrations ~85 to 95 min for C/EC-5-glucuronide and ~120 to 145 min for 3'OMe-C/EC-5-glucuronide (Table 5.4.) and were gradually eliminated from plasma within 24 h (Figure 5.6). C_{max} of C-5-glucuronide and EC-5-glucuronide were determined to be 165 and 158 µmol/L respectively in the high apple/GSE group and were significantly higher than low apple/GSE group (p=0.008 and p=0.005). For 3'OMe-C-glucuronide and 3'OMe-EC-glucuronide, C_{max} values were determined to be 35 and 13 µmol/L respectively for High apple/GSE group which were significantly higher than low apple/GSE group (p=0.0001 and p=0.0003) (Table 5.4). The dose dependence of plasma response for four major flavan-3-ol metabolites were further confirmed by significantly higher $AUC_{0\text{-}1440min}$ in high apple/GSE group compared to the low dose group with p=0.01, 0.006, 0.0004 and 0.0007 for C-5-glucuronide, EC-5-glucuronide, 3'OMe-C-glucuronide and 3'OMe-EC-glucuronide, respectively (Table 5.4).

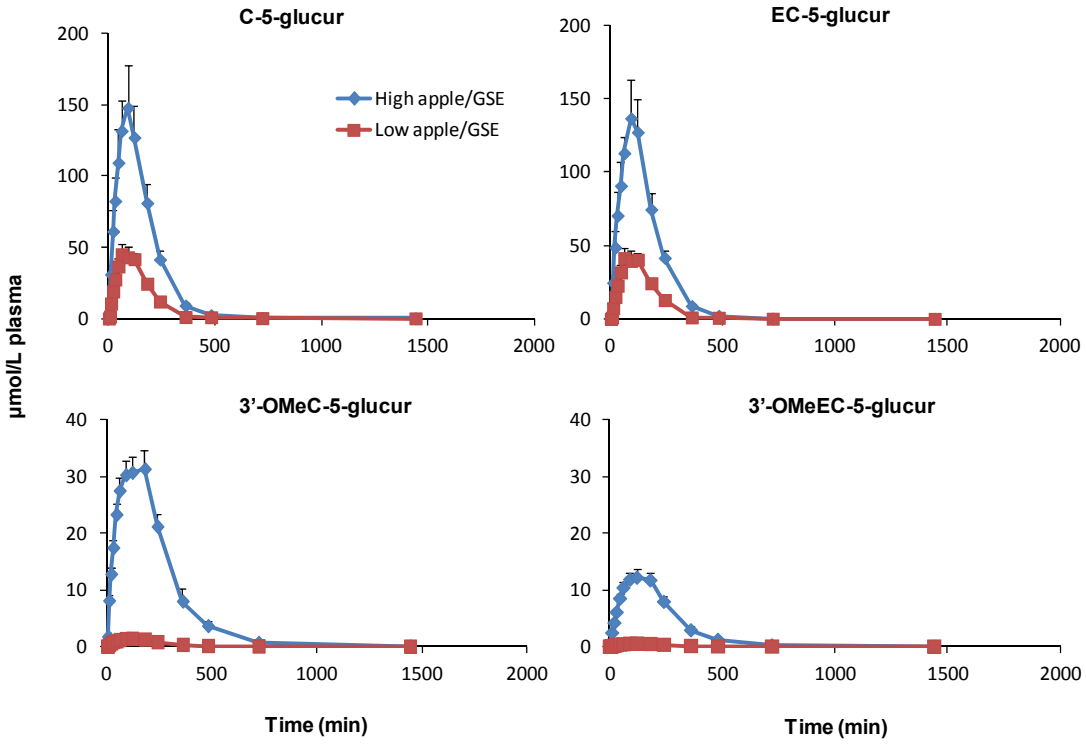


Figure 5.6. Plasma pharmacokinetic responses of flavan-3-ol metabolites from piglets treated with high or low dose of apple/GSE for 3 weeks.
No metabolites were detected in any piglets from control group (n=5).
Data was represented as mean ± SEM with n=6 piglets per treatment group.

Table 5.4. Pharmacokinetic parameters of flavan-3-ol metabolites in plasma from piglets treated with high or low dose of apple/GSE for 3 weeks.

	Parameters			
	Treatment	**$AUC_{0\text{-}1440min}$ (µmol/L*min)**	**C_{max} (µmol/L)**	**T_{max} (min)**
C-5-glucur	CNTL	ND	ND	ND
	High apple/GSE	27051.8 ± 4883.5*	165.8 ± 27.8*	90.0 ± 7.7
	Low apple/GSE	8273.5 ± 987.7	50.8 ± 8.3	85.0 ± 12.0
EC-5-glucur	CNTL	ND	ND	ND
	High apple/GSE	25282.0 ± 3950.5*	158.3 ± 24.4*	90.0 ± 7.8
	Low apple/GSE	7887.5 ± 985.8	47.1 ± 7.9	95.0 ± 12.0
3'-OMeC-5-glucur	CNTL	ND	ND	ND
	High apple/GSE	9662.8 ± 1128.4*	35.4 ± 3.2*	135.0 ± 21.6
	Low apple/GSE	399.2 ± 46.5	1.5 ± 0.1	120.0 ± 13.4
3'-OMeEC-5-glucur	CNTL	ND	ND	ND
	High apple/GSE	3609.2 ± 471.1*	13.4 ± 1.4*	145.0 ± 16.3
	Low apple/GSE	158.6 ± 22.6	0.6 ± 0.1	125.0 ± 12.0

No metabolites (ND) were detected in any piglets from control group (n=5).
Data was represented as mean ± SEM (n=6/treatment group).
'*' indicated significant difference between high and low dose groups with $p<0.05$.

Plasma pharmacokinetic profiles of Q-glucuronide and MeO-Q-glucuronide are illustrated in Figure 5.7. Plasma Q-glucur showed peak concentrations ~17 to 23 min and was gradually eliminated from the system by 24 h. There was no significant difference found between dose groups on C_{max} on Q-glucur, nor on $AUC_{0\text{-}1440min}$ (Table 5.5.). MeO-Q-glucuronide exhibited peak concentration at ~26 min for the high dose group and ~57 min for low dose group reached C_{max} of 3.33 and 1.13 nmol/L on high and low apple/GSE group, respectively (Table 5.5.).

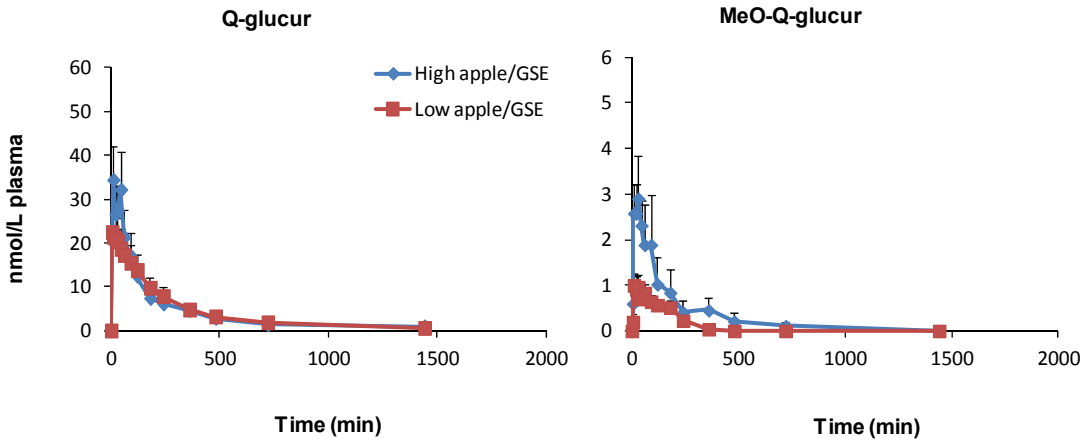


Figure 5.7. Plasma pharmacokinetic responses of quercetin metabolites after piglets were treated with high or low dose of apple/GSE for 3 weeks.
No metabolites were detected in any piglets from control group (n=5).
Data was represented as mean ± SEM (n=6 piglets/treatment group).

Table 5.5. Pharmacokinetic parameters of quercetin metabolites in plasma from piglets treated with high or low dose of apple/GSE for 3 weeks.

		Parameters		
	Treatment	$AUC_{0\text{-}1440min}$ (nmol/L*min)	C_{max} (nmol/L)	T_{max} (min)
Q-glucur	CNTL	ND	ND	ND
	High apple/GSE	6096.0 ± 830.0	36.8 ± 10.1	23.0 ± 8.0
	Low apple/GSE	6040.9 ± 1627.2	23.1 ± 7.0	17.0 ± 7.0
MeO-Q-glucur	CNTL	ND	ND	ND
	High apple/GSE	502.2 ± 253.6	3.3 ± 1.0	26.0 ± 8.0
	Low apple/GSE	168.1 ± 43.0	1.1 ± 0.0	57.0 ± 25.0

No metabolites (ND) were detected in any piglets from control group (n=5).
Data was represented as mean ± SEM (n=6/treatment group).
'*' indicated significant difference between high and low dose groups with $p<0.05$.

Plasma pharmacokinetic profiles of anthocyanidin-galactoside and –glucosides are illustrated in Figure 5.8. All anthocyanidin derivatives plasma concentrations reached a maximum between ~40 to 115 min after polyphenol ingestion (Table 5.6.). Our results were consistent with other reports demonstrating that anthocyanins were rapidly absorbed in both the gastric and upper small intestinal compartments of the GI tract as intact glycosides (Passamonti, Vrhovsek et al. 2003; Talavera, Felgines et al. 2003). C_{max} of individual anthocyanidin-galactosides ranged from 12 to 121 pmol/L in plasma in high bilberry group with Cy-galactoside being the highest. All five anthocyanidin-galactosides demonstrated significantly higher C_{max} in the high bilberry group than in the low bilberry group except for Mv-glucosdie only showing a trend toward significance (Table 5.6.). $AUC_{0\text{-}1440min}$ of Mv-, Dp- and Cy-galactoside in high bilberry group were significantly higher than those in low bilberry group. C_{max} of key anthocyanidin-glucosides including Pt-, Dp-, Pn- and Cy-glucoside ranged from 30 to 223 pmol/L in high bilberry group

which were found to be significantly higher than those in low bilberry group (Table 5.6.). C_{max} of Mv-glucoside only showed a trend toward to significance (p=0.08). Pt-, Pn- and Cy-glucoside in high bilberry group had significantly higher $AUC_{0\text{-}1440min}$ compared to low bilberry group. For Mv- (p=0.05) and Dp-glucoside (p=0.06), there was a trend toward significant differences in AUC (Table 5.6.). Overall, there was a dose-dependent increase in plasma response from high compared to low bilberry groups across all five key anthocyanidin-glucosides detected in plasma determined by $AUC_{0\text{-}1440min}$ and C_{max} (Table 5.6.).

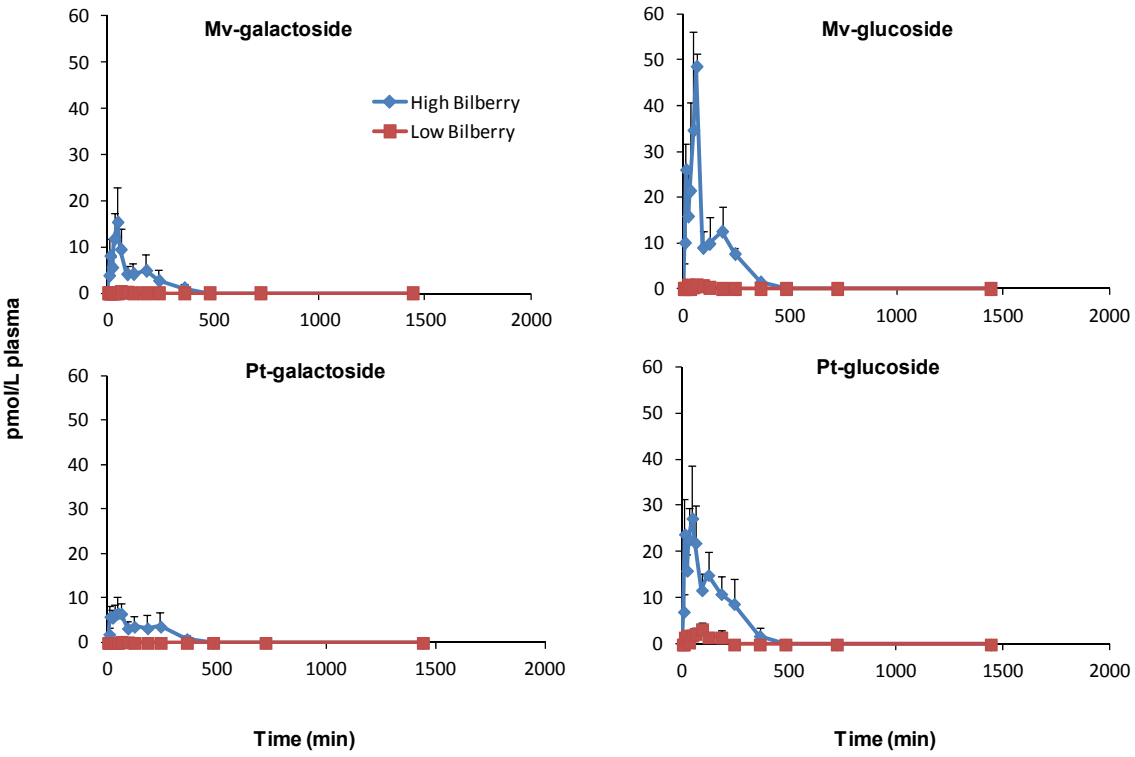


Figure 5.8. Plasma pharmacokinetic responses of major anthocyanidin glycosides including galactosides and glucosides after piglets were treated with high or low dose of bilberry extract for 3 weeks.
No metabolites were detected in any piglets from control group (n=5).
Data was represented as mean ± SEM with n=6 piglets per treatment group.

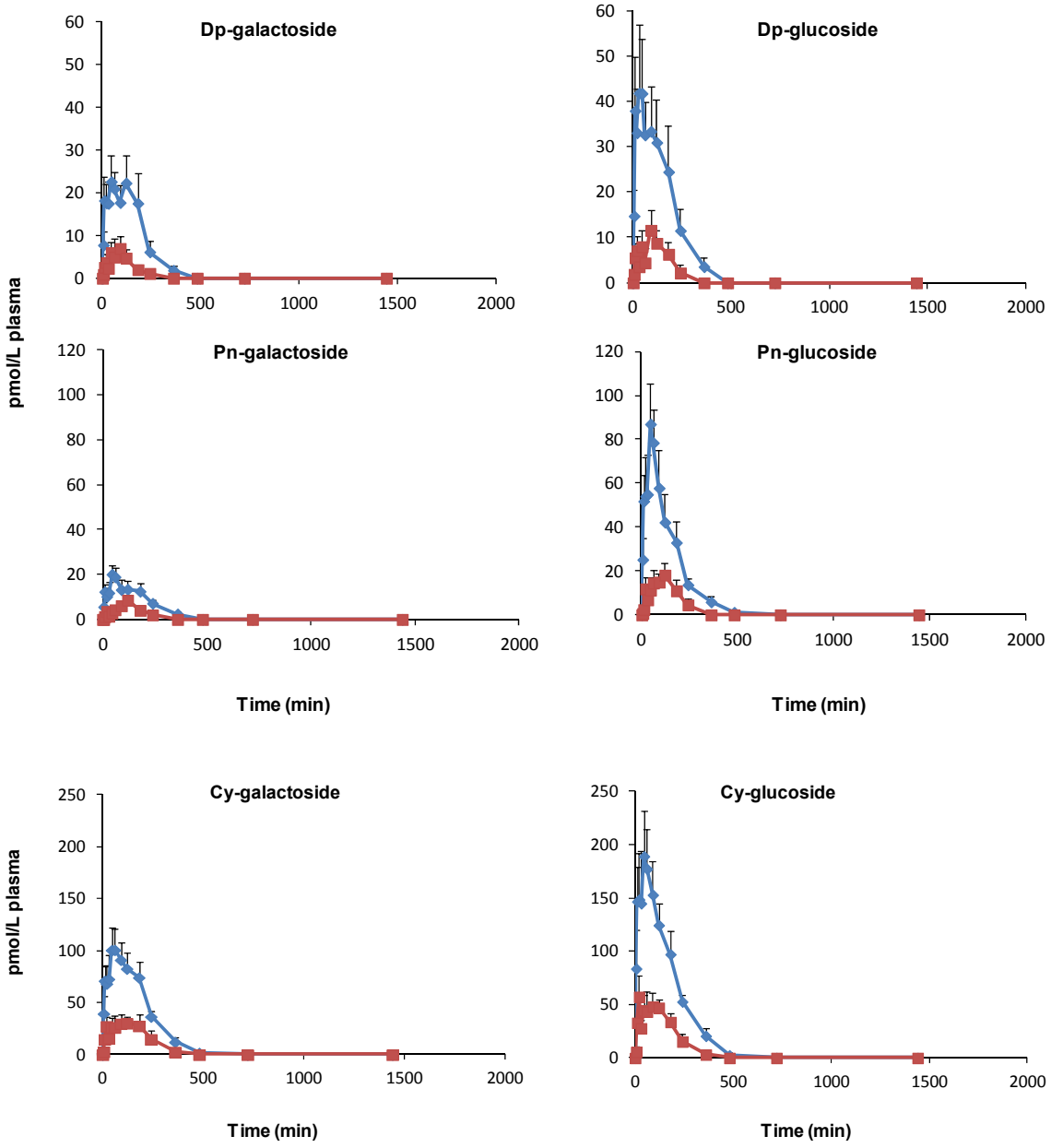


Figure 5.8. Continued.

Table 5.6. Pharmacokinetic parameters of anthocyanidin glycosides in plasma from piglets treated with high or low dose of bilberry extract for 3 weeks.

	Parameters			
	Treatment	**$AUC_{0\text{-}1440min}$ (pmol/L*min)**	**C_{max} (pmol/L)**	**T_{max} (min)**
Mv-galactoside	CNTL	ND	ND	ND
	High Bilberry	1700.3 ± 925.4*	17.2 ± 9.1*	58.0 ± 13.0
	Low Bilberry	9.3 ± 8.0	0.2 ± 0.2	NA
Pt-galactoside	CNTL	ND	ND	ND
	High Bilberry	1325.6 ± 816.6	12.7 ± 3.9*	83.0 ± 35.0
	Low Bilberry	59.7 ± 49.2	1.0 ± 0.6	NA
Dp-galactoside	CNTL	ND	ND	ND
	High Bilberry	4747.6 ± 1420.6*	32.0 ± 5.8*	99.0 ± 33.0
	Low Bilberry	931.6 ± 484.9	12.5 ± 4.2	65.0 ± 17.0
Pn-galactoside	CNTL	ND	ND	ND
	High Bilberry	3782.5 ± 1123.8^	23.3 ± 3.8*	57.0 ± 15.0
	Low Bilberry	1187.8 ± 504.3	9.9 ± 2.9	87.0 ± 18.0
Cy-galactoside	CNTL	ND	ND	ND
	High Bilberry	22170.1 ± 4437.9*	121.1 ± 18.9*	52.0 ± 15.0*
	Low Bilberry	7153.2 ± 2997.3	41.2 ± 11.7	115.0 ± 16.0
Mv-glucoside	CNTL	ND	ND	ND
	High Bilberry	4597.4 ± 1767.9^	60.6 ± 26.7^	44.0 ± 7.0
	Low Bilberry	58.6 ± 37.1	0.9 ± 0.6	40.0 ± 12.0
Pt-glucoside	CNTL	ND	ND	ND
	High Bilberry	4251.3 ± 1351.2*	39.3 ± 12.6*	52.0 ± 15.0
	Low Bilberry	376.4 ± 181.9	4.8 ± 1.6	85.0 ± 29.0
Dp-glucoside	CNTL	ND	ND	ND
	High Bilberry	7905.5 ± 2527.0^	57.3 ± 13.2*	62.0 ± 19.0
	Low Bilberry	1697.0 ± 696.4	18.1 ± 3.0	79.0 ± 18.0
Pn-glucoside	CNTL	ND	ND	ND
	High Bilberry	12553.8 ± 3586.5*	93.27 ± 18.5*	44.0 ± 8.0
	Low Bilberry	3044.2 ± 1385.4	23.00 ± 6.3	82.0 ± 17.0
Cy-glucoside	CNTL	ND	ND	ND
	High Bilberry	35391.3 ± 7847.4*	223.5 ± 36.8*	40.0 ± 9.0
	Low Bilberry	10188.4 ± 3307.6	78.5 ± 19.8	52.0 ± 19.0

Transformed data: Mv-galactosid ($AUC_{0\text{-}1440min}$, C_{max} and T_{max}; Pt-galactoside ($AUC_{0\text{-}1440min}$); Pn-galactoside ($AUC_{0\text{-}1440min}$), Cy-galactoside ($AUC_{0\text{-}1440min}$); Mv-glucoside (C_{max} and T_{max}), Pt-glucoside ($AUC_{0\text{-}1440min}$).

No metabolites (ND) were detected in any piglets from control group (n=5).

Data was represented as mean ± SEM with n=6 piglets per treatment group.

'*' indicated significant difference between high and low dose groups with $p<0.05$.

'^' indicated a trend toward significant difference between high and low dose groups with $p<0.01$.

Regional differences in brain distribution of polyphenols from apple/GSE or bilberry treated piglets

In order to determine the potential for targeting of individual brain regions, the polyphenol metabolite content across six brain regions was analyzed for both high and low dose group. Within the high dose apple/GSE group (Figure 5.9.), cerebellum showed significantly higher deposition levels of C-5-glucuronide and 3'OMe-C/EC-glucuronide than all other brain regions ($p<0.0001$). The same pattern was observed in the low dose apple/GSE group in that cerebellum showed significantly higher deposition levels of C-5-glucur and 3'OMe-C/EC-glucuronide than all other brain regions ($p<0.0001$). For EC-5-glucur, however, cerebellum showed significantly higher deposition level compared to hippocampus ($p=0.02$) and hypothalamus ($p=0.01$), but not to brain stem, frontal cortex and amygdala for both dose groups.

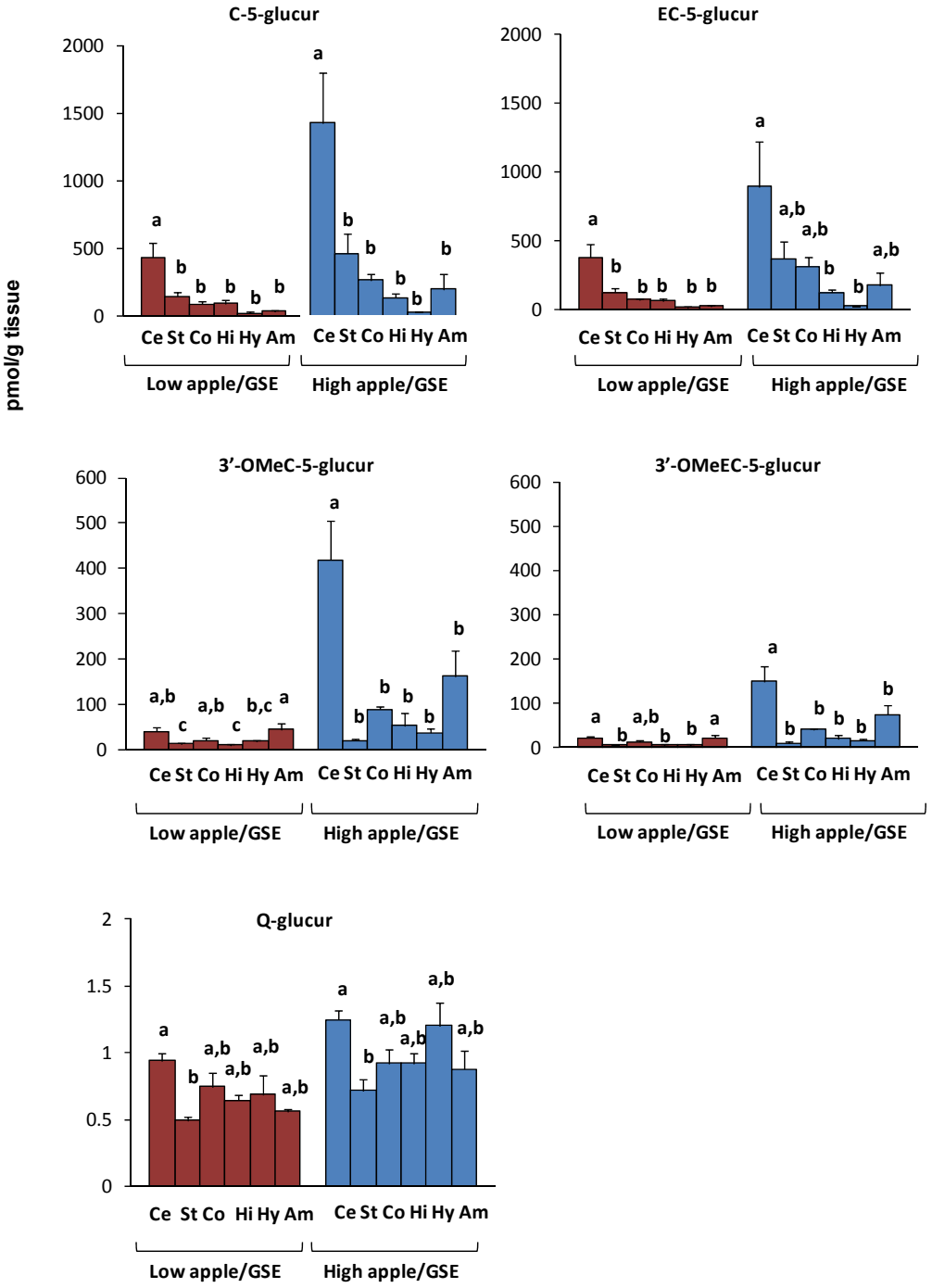


Figure 5.9. Regional brain accumulation of flavan-3-ol and quercetin metabolites from piglet treated with high or low doses of apple/GSE for 3 weeks.
Different letters represented significant difference among brain regions within high or low dose group.
Data was presented as mean ± SEM (n=6 per brain region).

Similarly, the levels of anthocynidin glycosides were compared across six different brain regions within the high and low bilberry group (Figure 5.10.). Within low dose bilberry group, Mv-galactoside and Mv-glucoside were detected in cerebellum, brain stem and frontal cortex but there was no significant difference between regions. Pt-galactoside was not detected in any brain region in the low dose bilberry group whereas Pt-glucoside was only detected in brain stem in one piglets and therefore, did not significantly differ from other regions ($p=0.46$). Dp-galactoside and Dp-glucoside were detected in brain stem but did not significantly differ from other regions. Pn-galactoside and Pn-glucoside was detected in brain stem which significantly differed from other regions ($p=0.02$).There were no regional differences in Pn-galactoside. Cy-galactoside was detected in cerebellum, brain stem and frontal cortex but no significant differences were observed between regions ($p=0.08$). Cy-glucoside was detected in cerebellum and brain stem and there was no significant difference observed between regions.

When comparing brain deposition levels across brain regions within high bilberry group (Figure 5.10.), cerebellum was observed to have significantly higher deposition levels of Mv-galactoside, Mv-glucoside, Pt-galactoside, Pt-glucoside, Pn-galactoside, Cy-galactoside and Cy-glucoside than other regions (all with $p<0.0001$). With Pn-glucoside, cerebellum was determined to have a significantly higher deposition level than all other regions and brain stem had significantly higher deposition level than hippocampus, hypothalamus and amygdala but did not differ from frontal cortex (Figure 5.10.). There

was no significant difference shown across regions in Dp-galactoside (p=0.11) or Dp-glucoside (p=0.11).

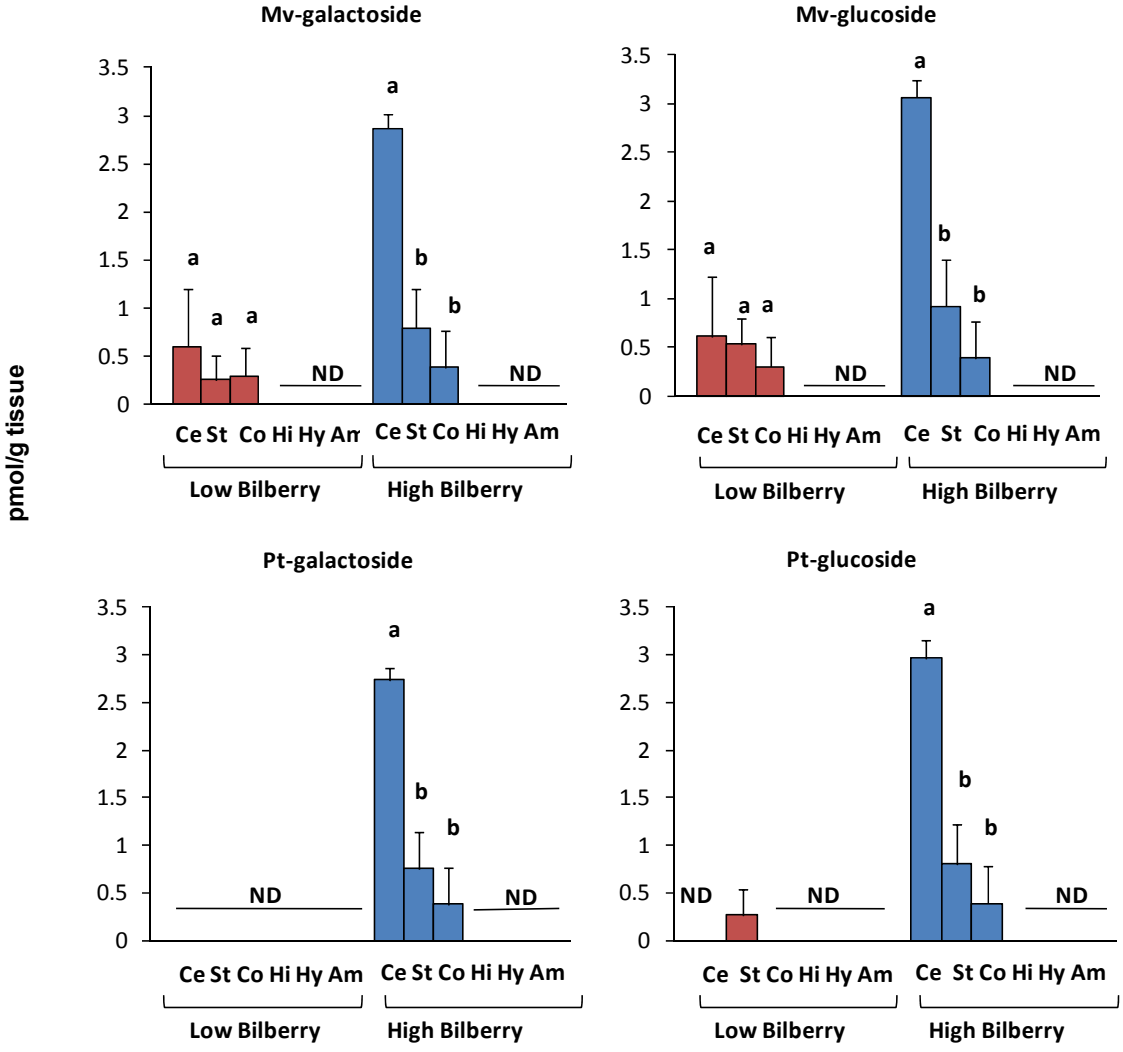


Figure 5.10. Regional brain accumulation of anthocyanidin glycosides from piglet treated with high or low doses of bilberry extract for 3 weeks.
Different letters represented significant difference among brain regions within high or low dose group.
Data was presented as mean ± SEM (n=3 per brain region).
ND= non detected. LOD=0.40 pg on column for Mv-3-glucoside and 0.47 for Cy-3-glucoside.

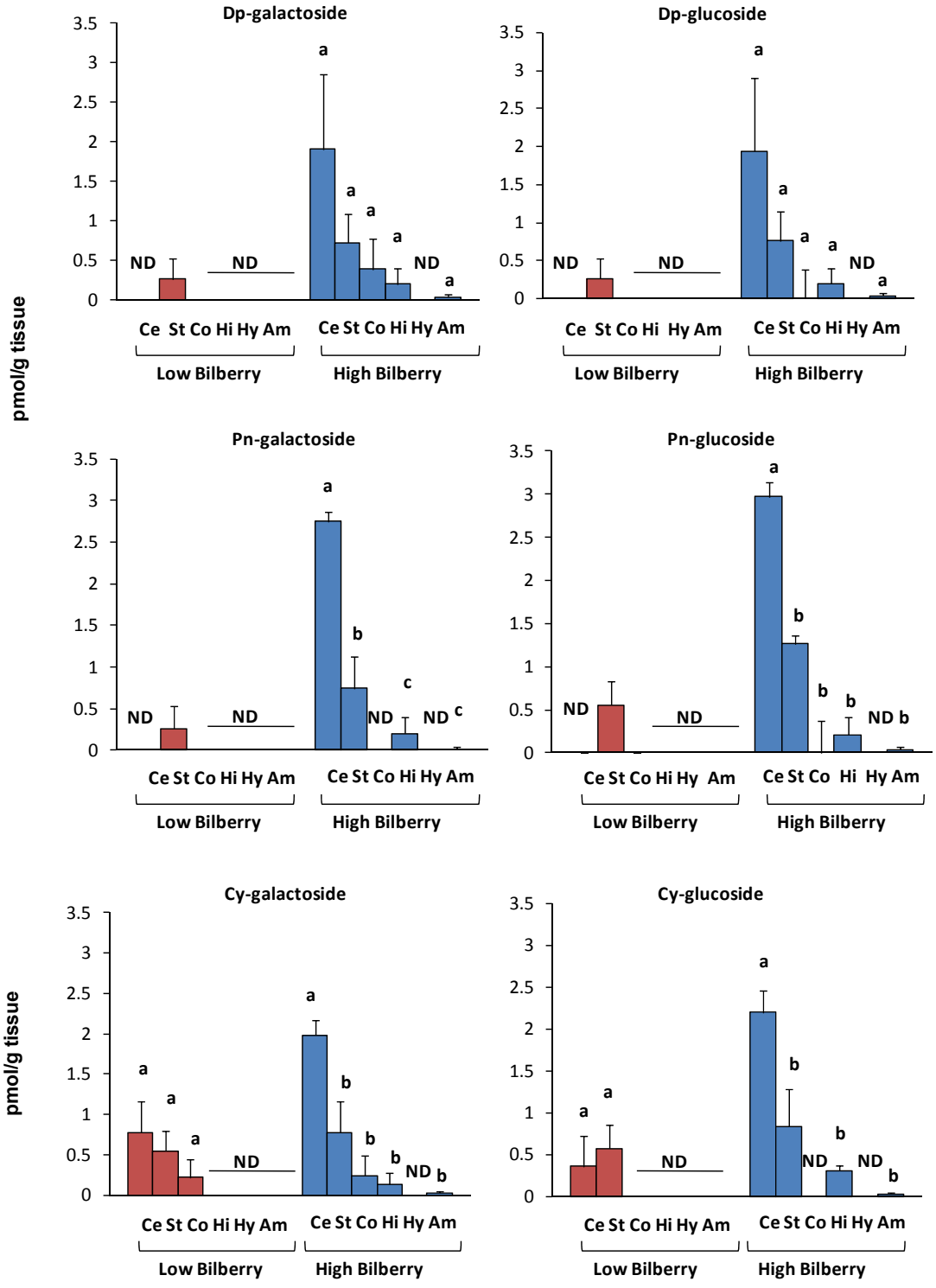


Figure 5.10. Continued.

Dose effect on brain distribution of polyphenols from apple/GSE or bilberry treated piglets

When comparing polyphenol metabolite levels in brain tissues between high and low doses within the same treatment (Figure 5.11.), there was a dose response dependent increase of C-5-glucur content in cerebellum ($p=0.04$), frontal cortex ($p=0.01$) and a trend in brain stem ($p=0.09$). On EC-5-glucur, the dose response was significant in frontal cortex ($p=0.02$) and showed a trend for increase with high apple/GSE treatment in hippocampus ($p=0.07$). For 3'OMe-C-glucur, there was a dose response in cerebellum ($p=0.01$) and frontal cortex ($p<0.0001$) and a trend in hypothalamus ($p=0.08$). For 3'OMe-EC-glucur, the dose response was significant in cerebellum ($p=0.01$), frontal cortex ($p<0.0001$), hypothalamus ($p=0.05$) and showed a trend in amygdala ($p=0.08$).

The dose dependent increase in brain tissue content was significant for Q-glucur in cerebellum ($p=0.01$), brain stem ($p=0.03$), hippocampus ($p=0.01$) and hypothalamus ($p=0.04$) shown in Figure 5.11. Interestingly, there was no quantifiable MeO-Q-glucur found in brain extracts from apple/GSE treated piglets.

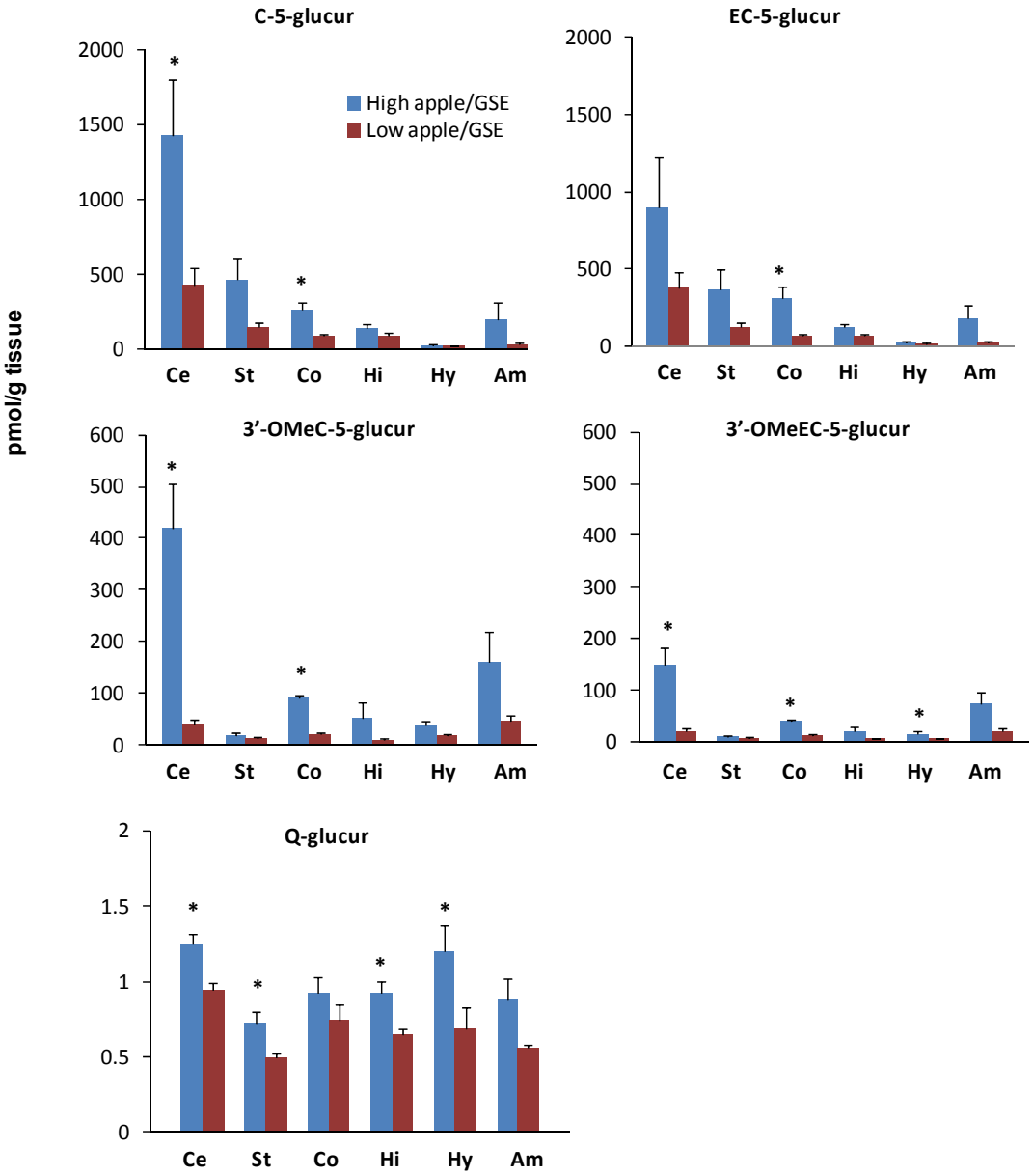


Figure 5.11. Dose differences of brain deposition of flavan-3-ol and quercetin metabolites from piglet treated with high and low doses of apple/GSE for 3 weeks. Asterisk represented significance between high and low dose groups within each brain region.
Abbreviations: cerebellum (Ce), brain stem (St), frontal cortex (Co), hippocampus (Hi), hypothalamus (Hy) and amydgala (Am).
Data was presented as mean ± SEM (n=6 per brain region).

For bilberry anthocyanins, a significant dose dependent increase was only observed in cerebellum for select anthocyanidin-galactosides (Figure 5.12.), including Mv-galactoside ($p=0.02$), Pt-galactoside ($p=0.002$), Pn-galactoside ($p=0.002$), and Cy-galactosides ($p<0.04$) but not Dp-galactoside ($p=0.18$). Dose dependence in cerebellum was also significant for Mv-glucoside ($p=0.0153$), Pt-glucoside ($p=0.0047$), Pn-glucoside ($p=0.038$) and Cy-glucosides ($p=0.0118$) but not Dp-glucoside ($p=0.1845$). In other brain regions including brain stem, frontal cortex, hippocampus, hypothalamus and amydgala, there was no significant dose effect between the high and low dose groups. This is likely due to the no detectable anthocyanins in the low bilberry group that results in the statistically non significance between groups.

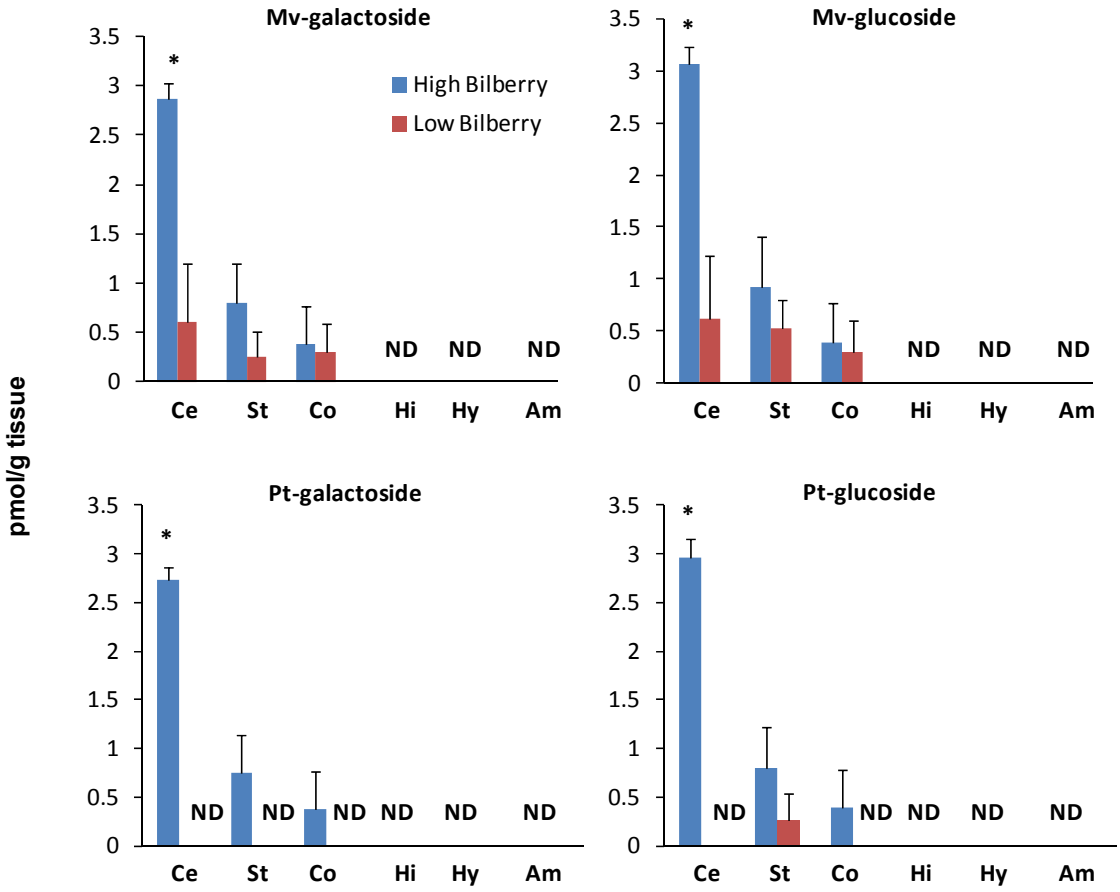


Figure 5.12. Dose differences of brain deposition of anthocyanidin glycosides from piglet treated with high and low doses of bilberry extract for 3 weeks.
Asterisk represented significance between high and low dose groups within each brain region.
Data was presented as mean ± SEM (n=3 per brain region).
ND= non detected.
LOD=0.40 pg on column for Mv-3-glucoside and 0.47 pg for Cy-3-glucoside.

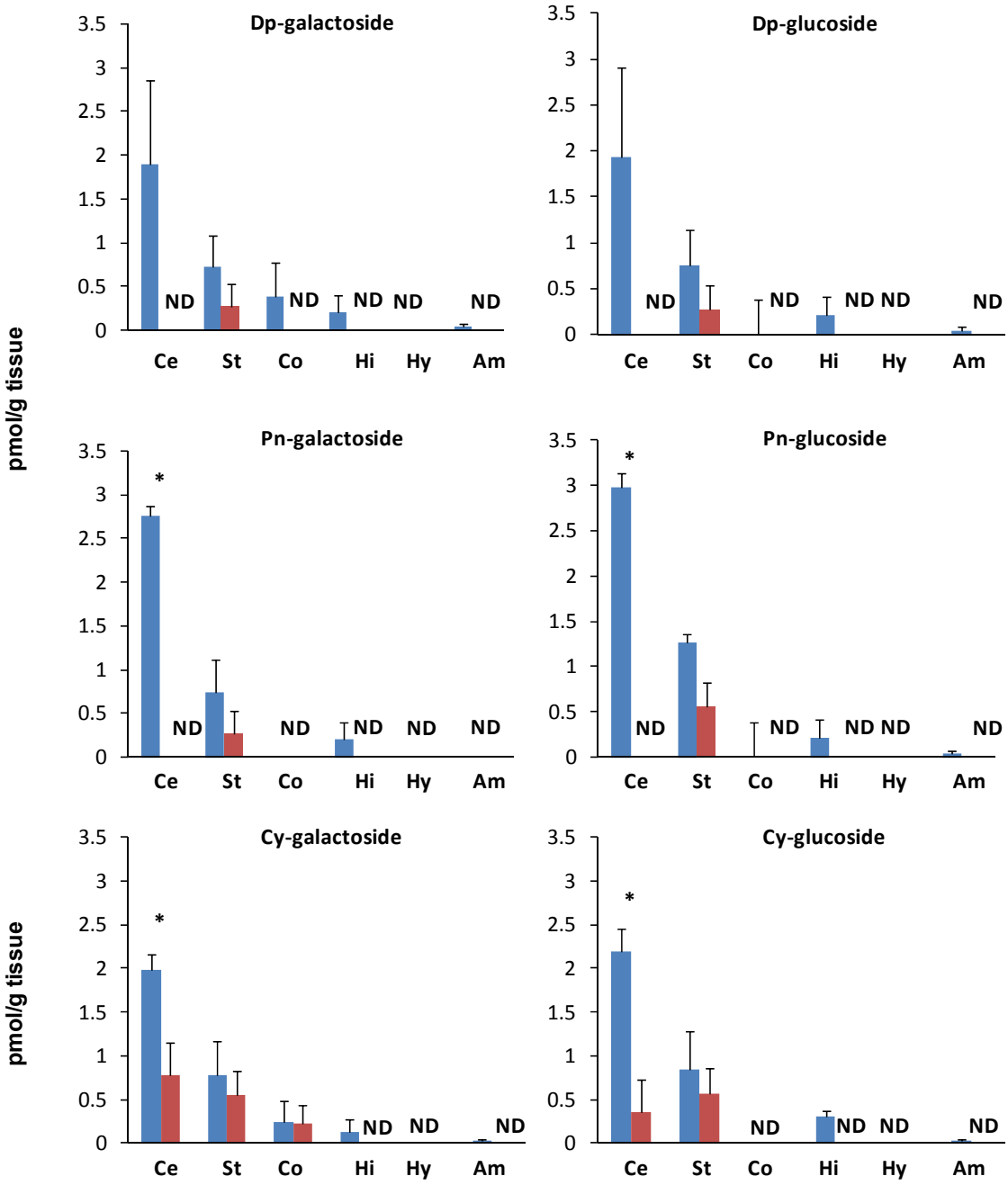


Figure 5.12. Continued.

5.4 Discussion

Limited information is currently available on polyphenol bioavailability in infants or children. Furthermore, very little is known regarding specific distribution patterns of polyphenol metabolites in the brain. With growing interest in understanding the role polyphenols may play in promoting healthy brain development and function, the current study was designed to investigate both plasma pharmacokinetics and regional brain deposition of key polyphenol metabolites in young piglets treated with apple/GSE or bilberry extract at two dose levels.

The most abundant polyphenol form in grape seed and apple fruit or extract are the proanthocyanidins including C and EC in monomeric and polymeric forms (Table 5.2.). The detection of flavan-3-ol metabolites in piglet plasma and brain extracts in this study is consistent with previous findings in Sprague-Dawley (SD) rats (Wang, Ferruzzi et al. 2012). What we found in the SD rat plasma and brain tissue dosed for 10 days with 17 or 28.3 mg/kg body weight of monomeric or polymeric C/EC fractions of GSE, the same C and EC metabolites as those found in pigs suggesting that the similar C and EC metabolites are generated by both species and distributed in the brain. The monomeric fraction of C/EC improved cognitive function in Tg22576 Alzheimer's disease (AD) model mice (Wang, Ferruzzi et al. 2012). The presence of similar C and EC metabolites in piglet plasma and brain tissue suggest that these compounds may be candidates for subsequent studies focused on brain specific benefits in young healthy animal models.

We have previously found Q-3-glucur in plasma and brain extracts from SD rats chronically treated with Cabernet Sauvignon for 10 days. Q-3-glucur was found to be the compound responsible for reducing β-amyloid peptides in a primary neuron culture from Tg2576 Alzheimer's model mice (Ho, Ferruzzi et al. 2013). However, in the current study, a Q-glucur with unknown position of glucuronidation was found in piglets suggesting the possibility of a different metabolite profile between species. Many studies have demonstrated that the position sites for glucuronidation on Q-glucur were 3, 4' or 7 positions. For example, Gee et al. reported that in a SD rat gut model, Q-3-glucur and Q-7-glucur were detected in mucosal tissue and within serosal compartment (Gee, DuPont et al. 2000). Moon et al. detected Q-3-glucur and Q-4'-glucur in rat plasma 20 min after an oral dose of 250mg/kg body weight of pure quercetin (Moon, Tsushida et al. 2001). In human feeding trials, the main quercetin metabolites detected in human plasma were Q-3-glucur (Day and Williamson 2001; Janisch, Williamson et al. 2004). However, there are no data on quercetin metabolites in pigs since the majority of the studies deconjugated metabolites to the aglycon by incubating samples with β-glucuronidase (Ader, Wessmann et al. 2000; Lesser, Cermak et al. 2004; de Boer, Dihal et al. 2005; Bieger, Cermak et al. 2008). Our data demonstrated the first time the major quercetin metabolite detected in pig plasma and brain extract was not Q-3-glucur and may therefore be speculate to position and suggest further study.

One consensus in the discussion of neuroprotective properties of polyphenols is that polyphenols need to cross the blood-brain barrier and localize in brain tissues to exert

their benefits. Most studies used rodent models to determine polyphenol concentrations in brain (Percival 2005; Ferruzzi, Lobo et al. 2009; Del Bo, Ciappellano et al. 2010; Juan, Maijo et al. 2010; Serra, Macia et al. 2012) since human brains are nearly impossible to obtain. Recently, there has been an increasing numbers of reports using larger animals for this purpose. Kalt et al. found ~0.66 and 0.88 pmol/g of anthocyanins in pig cerebellum and frontal cortex after the pigs were fed on 4% blueberry diet for 4 weeks (Milbury and Kalt 2010). Their follow-up study detected ~0.7, 0.87, 0.92 pmol/g of anthocyanins and glucuronides combined in frontal cortex, cerebellum and midbrain after pigs were fed on 2% blueberry diet for 8 weeks (Milbury and Kalt 2010). Our data on regional brain levels of anthocyanidin derivatives were estimated between 0 to 3 pmol/g depending on brain regions and compounds after piglets were dosed with bilberry extract for 3 weeks. Furthermore, the brain tissues used in the current study were harvest after the piglets were perfused with ice-cold saline to be obtained blood-free while this may not be the case in previous studies where tissues may have only been rinsed and not perfused (Milbury and Kalt 2010). Residual blood in tissues has been shown to greatly influence polyphenol concentrations and often need to be corrected for accurate determination (Friden, Ljungqvist et al. 2010).

5.5 Conclusion

To our knowledge, only limited information exists on regional brain distribution of anthocyanins, flavan-3-ols, flavonols and their metabolites. In the present study, a swine model which provided adequate brain tissue to study the regional differences in levels

of key polyphenols from apple/GSE and bilberry was applied to characterize flavan-3-ol, quercetin metabolites and anthocyanidin glycoside kinetics in plasma and distribution in brain tissues. Using both a high or low dose of apple/GSE blend or bilberry extracts for 3 weeks, we confirmed similar flavan-3-ol metabolites in blood and brain tissues to those reported in rodent models (Wang, Ferruzzi et al. 2012). Furthermore, flavan-3-ol metabolites and quercetin metabolites were found in all six regions regardless the dose suggesting that these polyphenols were able to cross blood-brain-barrier even in a low dietary dose range. In contrast, anthocyanin derivatives were not able to be detected in brain regions in a low dose treatment suggesting the levels were below LOD (0.4 pg on column for both Cy-3- and Mv-3-glucoside). Further examination on brain deposition of polyphenols across brain region demonstrated that cerebellum may be a primary site of accumulation for flavan-3-ol, quercetin metabolites and anthocyanidin derivatives. Future efficacy studies might need to consider the differential deposition of polyphenols in brain regions to optimize the dose consideration.

CHAPTER 6. CONCLUSION AND FUTURE DIRECTIONS

6.1 Overall Conclusion

Alzheimer's disease (AD) is projected to affect 5 billion Americans by the year 2050 (Hebert, Weuve et al. 2013). Its severity and prevalence called for immediate attention for research to find a cure. Epidemiological studies have correlated the consumption of fruits and vegetables and other beverages rich in polyphenols to the decreased risk for age-related neurodegenerative disorders (Orgogozo, Dartigues et al. 1997; Truelsen, Thudium et al. 2002; Scarmeas, Stern et al. 2006; Letenneur, Proust-Lima et al. 2007; Scarmeas, Stern et al. 2009). There is increasing evidence showing the benefits of flavonoids including GSE (Wang, Ho et al. 2008; Wang, Thomas et al. 2009), grape juice, wine (Wang, Ho et al. 2006; Ho, Chen et al. 2009) and resveratrol (Marambaud, Zhao et al. 2005) in *in vitro* and *in vivo* AD mouse model supporting their Aβ-lowering effects. Since neuroprotection and preservation are the major actions of polyphenols to counteract neurodegeneration, there has been growing interests in the effects of polyphenols on neuron development in infants and young children. However, the evidence of the effect of polyphenols on healthy young groups is lacking. In order to gain insights into the therapeutic effects of polyphenol-rich fruits extracts in brain health in the elderly and the young, it is essential to characterized the bioactives, their

absorption profiles and factors that influence bioavailability. This dissertation is aimed to fill that knowledge gap with a special focus on bioavailability of polyphenols and their implications for brain health.

The overall objective of this research was to study the bioavailability, metabolism and brain distribution of the major flavonoids and stilbenoid from polyphenol-rich fruit products and extracts including GSE, Concord grape juice, resveratrol, apple and bilberry extracts. The absorption and brain profiles of SGP (mixture of GSE, juice and resveratrol) were evaluated as functions of diabetogenic diet and diabetic condition in rodent models. To extend the possible neuroprotection effects to the young, a weaning piglet model was used to investigate bioavailability of GSE/apple and bilberry extracts and their brain deposition levels. Regional brain accumulation differences were also determined.

In summary, Chapter 3 concluded that similar metabolites were found in plasma and brain tissues when polyphenol-rich extracts were treated individually or simultaneously as SGP in a Sprague-Dawley rat model. The impact of 3 weeks of diabetogenic diet feeding on polyphenol absorption and brain deposition levels were minimum. Based on the data obtained from the first study, fat composition in diet can be eliminated as a confounding factor in regard to polyphenol absorption. Following upon on the first study, Chapter 4 highlights the effects of the diabetic condition on the bioavailability of the same polyphenolic preparation (SGP) in a Zucker diabetic rat model. Similar polyphenol metabolite profiles were found in both healthy SD and diabetic rat models. We concluded that the diabetic condition significantly decreased plasma bioavailability

and brain deposition of flavan-3-ol, quercetin and resveratrol metabolites. We suspected that the decreased polyphenol absorption was due to excessive urinary excretion in Zucker diabetic rats. Taken together, Chapter 3 and 4 suggested that 3 weeks of diabetogenic diet may minimally affected polyphenol bioavailability, however, the diabetic condition drastically decreased polyphenol absorption and brain distribution possibly to due the heavy excretion in urine. Interestingly, after normalized by SGP dose, flavan-3-ol metabolites demonstrated high brain concentrations in both healthy Sprague-Dawley and diabetic rat models compared to other polyphenol metabolites including quercetin, resveratrol metabolites and anthocyanins. This is exciting since this data indicted that flavan-3-ol metabolites had preferable assess into the brain compared to other polyphenol sources suggesting that flavan-3-ol metabolites are promising candidates for AD prevention.

Chapter 5 used a piglet model to represent young children to investigate the bioavailability of GSE/apple and bilberry extracts in either a physiological or a pharmacological dose. Flavan-3-ol, quercetin metabolites and anthocyanins were the major bioactives circulating in the blood and targeted brains even on a physiological dose which was determined to be achievable by supplements. Additionally, differences on polyphenol accumulation in brain regions with cerebellum being the highest on deposition levels of flavonoid metabolites suggested that these bioactives showed preferable access into cerebellum.

In conclusion, bioavailability of polyphenols from SGP (GSE, Concord grape juice and resveratrol) derived metabolites (flavan-3-ols, quercetin, resveratrol and

anthocyanins) can be negatively affected by the diabetic condition but not by diabetogenic diet under the treatment conditions used in this research. This observation implied that disease condition should be given great consideration when recommending the dosage of dietary supplements to patients. On the other hand, the same classes of flavonoids metabolites derived from GSE/apple and bilberry extracts were found bioavailable in a young piglet model. Concentrations in plasma and brain were found to be dose dependent for flavan-3-ol metabolites and anthocyanins but not for quercetin metabolites. Differential deposition of flavonoids in brain regions suggesting that cerebellum is a preferred site for accumulation.

6.2 Future Directions

Our data demonstrated the first time that the diabetic condition negatively influences flavonoid and stilbenoid absorption. While the data are promising, the speculation on the mechanism of whether the impaired absorption of flavonoids in ZDF rats is entirely due to excessive excretion through urine needs further investigation. It has been shown that impaired insulin signaling can change the expression of drug metabolizing enzymes which are responsible for glucuronidation and sulfation on polyphenol metabolites such as UDP-glucuronosyltransferase and sulfotransferases (Kim and Novak 2007). The change of activities in phase II metabolizing enzymes is very likely to alter the generation of conjugated polyphenol metabolites. It will be interesting to further explore whether or not the function of UDP-glucuronosyltransferase, sulfotransferases and COMT are compromised in diabetic

animals versus non-diabetic ones. Since the lower plasma and brain levels of polyphenols is the net balance between poor absorption and high excretion, further investigation needs to address the intestinal absorption to establish the mechanism of action.

Our data on the bioavailability of GSE/apple and bilberry extracts in young piglets warrants further functionality study to determine whether these bioactives help improve brain development regarding cognitive functions and learning abilities in young children. It was demonstrated for the first time that there is differential brain deposition of polyphneols and that flavonoids from studied fruit extracts preferably accumulate in cerebellum. Since there is a growing consensus that cerebellum is involved in memory, learning and cognition (Lalonde and Strazielle 2012; Wu and Hallett 2013), our data support further investigation on the benefits of flavonoids on child brain development and prevention or treatment of cerebellar disorders.

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VITA

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Education

2006-2008 **M.Sc., Rutgers, The State University of New Jersey, New Brunswick, NJ, USA**
Food Science

2001-2005 **B.Sc., National Taiwan University (NTU), Taipei, Taiwan**
Major: Animal Science, Minor: Food Science

Research Interests

2008-Present **Graduate Researcher**, Phytochemical Bioavailability Laboratory
Department of Nutrition Science & Food Science, Purdue University
Advisors: Elsa M. Janle, Ph.D. & Mario G. Ferruzzi, Ph.D.

Project: Brain Bioavailability of Polyphenols: Implications for Delivery of Brain Health Benefits

- Identified & characterized novel polyphenolic derivatives & metabolites (flavan-3-ols, flavonols, anthocyanidins & resveratrol) in foods & biological samples by LC-MS & LC-MS^2, which have proven beneficial to ameliorate Alzheimer's disease in a rodent model (in collaboration with Dr. Giulio M. Pasinetti at Mount Sinai Medical School)
- Developed pharmacokinetic model of phytochemicals on *in vivo* and *in vitro* models to recommend optimal doses for efficacy studies applied to infant product development & treatments for neurodegenerative diseases
- Quantified changes induced by phytochemical consumption in neurotransmitters by LC-ECD & in-brain morphology using software *Image J* for MRI data analysis
- Experienced in animal surgery techniques, i.e. jugular catheter & brain microdialysis probe implantation to study brain localization of phytochemicals

2006-2008 **Graduate Researcher**, Nutraceutical Nanoencapsulation Laboratory
Department of Food Science & Chemical Biology, Rutgers University
Advisors: Qingron Huang, Ph.D. & Mou-Tuan Huang, Ph.D.

Thesis: Anti-Obesity Effects of Resveratrol, Black Tea Extract, & Caffeine in Mice

- Constructed nanoemulsion formulation stabilized by biopolymers (soybean lecithin) to study the application on flavor & nutracuetical encapsulation for development of controlled-release system
- Characterized the nanoemulsions by dynamic light scattering & inverted optical microscope to analyze product properties & increase product stability
- Performed ELISA on proinflammatory markers (IL-1β & IL-6) to identified anti-inflammatory effects of polyphenols in a rodent model

Publications

Chen TY, Hargett K, Shin D, Song BJ, Cooper B, Jouni Z, Feruzzi MG, Janle EM. Plasma & Regional Brain Bioavailability of Polyphone-Rich Products, Apple/Grape Seed Extracts & Bilberry, in a Young Swine Model. (In preparation)

Chen TY, Wu QL, Simon JE, Talcott ST, Wang J, Ho L, Pasinetti GM, Shin D, Todd G, Cooper B, Feruzzi MG, Janle EM. Influence of Diabetes on Plasma Pharmacokinetics & Brain Bioavailability of Grape Polyphenols in the Zucker Diabetic Fatty Rat Model. (In preparation)

Wang J, Tang C, Ferruzzi MG, Gong B, Song BJ, Janle EM, **Chen TY**, Cooper B, Varghese M, Cheng A, Freire D, Roman J, Nguyen T, Ho L, Talcott ST, Simon JE, Wu QL, Pasinetti GM. Role of Standardized Grape Polyphenol Preparation as a Novel Treatment to Improve Synaptic Plasticity through Attenuation of Features of Metabolic Syndrome. (Under review)

Ho L, Ferruzzi MG, Janle EM, Wang J, Gong B, **Chen TY**, Lobo J, Cooper B, Wu QL, Talcott ST, Percival SS, Simon JE, Pasinetti GM. Identification of Brain-Targeted Bioactive Dietary Quercetin-3-O-Glucuronide as a Novel Intervention for Alzheimer's Disease. FASEB J. 2013 Feb; 27(2):769-81

Conference Posters

Influence of Diabetes on Plasma Pharmacokinetics & Brain Bioavailability of Grape Polyphenols in the Zucker Rat Model; Experimental Biology, Expected April 2013, Boston, MA

Bioavailability & Brain Deposition of Proanthocyanidin, Anthocyanin & Flavonoid in "*Combi-Phenol*" Treated
Rats on High Fat or Low Fat Diet; Experimental Biology, April 2012, San Diego, CA

Bioavailability & Brain Distribution of Anthocyanin & Quercetin Metabolites from Grape Juice & Wine;
Experimental Biology, April 2011, Washington D.C.

Identification & Characterization of Brain-Targeting Grape-Derived Polyphenolics: Implications in Alzheimer's Disease Prevention & Therapy; 40th Annual Meeting of Neuroscience, November 2010, San Diego, CA